

21 March 2014 | \$10

Science

Shocking Black Hole in M83

EDITORIAL

- 1289 *Exxon Valdez: 25 Years Later*
Marcia McNutt

NEWS OF THE WEEK

- 1294 A roundup of the week's top stories

NEWS & ANALYSIS

- 1296 First Wrinkles in Spacetime Confirm Cosmic Inflation
1298 Hints of Detente Between NSF and Republicans
1299 Irreproducibility Dogs New Reprogramming Method
1301 U.S. and Mexico Unleash a Flood Into Colorado Delta

NEWS FOCUS

- 1302 A War Within a War
1306 The Vigilante

LETTERS

- 1311 When Science-Based Management Isn't
K. A. Artelle et al.
U.S. Policy Impedes Innovators
A. Conwill et al.
Supporting the Scientific Diaspora
S. de Silva

1313 CORRECTIONS AND CLARIFICATIONS

BOOKS ET AL.

- 1314 Social Networks and Popular Understanding of Science and Health
B. G. Southwell, reviewed by E. Brennan
1315 Discoveries
N. Thomas et al., curators, reviewed by D. Dixon

POLICY FORUM

- 1316 Carbon Market Lessons and Global Policy Outlook
R. G. Newell et al.

PERSPECTIVES

- 1318 Testing the Limits of Accretion
A. King
>> Report p. 1330
1319 Nanoframe Catalysts
J. R. Greer
>> Report p. 1339
1320 Unlocking Ancient Protein Palimpsests
E. Cappellini et al.
1322 RIPK3 Takes Another Deadly Turn
J. Zhang and F. K.-M. Chan
>> Report p. 1357
1323 Epstein-Barr Virus Turns 50
P. M. Lieberman
1325 Flow of Control in Networks
J.-P. Onnela
>> Report p. 1373
1326 Counting the Ways to Decode Dynamic Signals
R. Wollman
>> Research Article p. 1329

REVIEW

- 1328 Virulence and Pathogenesis of HIV-1 Infection: An Evolutionary Perspective
C. Fraser et al.
Review Summary; for full text:
<http://dx.doi.org/10.1126/science.1243727>

CONTENTS continued >>



page 1302



pages 1323

ON THE WEB THIS WEEK

>> Science Podcast

On this week's show: human scent discrimination pegged at 1 trillion odors and a roundup from our daily news site.

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COVER

Composite image from NASA's Hubble Space Telescope of the spiral galaxy M83 (diameter ~70% that of the Milky Way). Embedded near the center of this image is a stellar-mass black hole that bombards its surroundings with kinetic energy through hugely powerful jets. See pages 1318 and 1330.

Image: NASA, ESA, and the Hubble Heritage Team (STScI/AURA); W. P. Blair (STScI/JHU)

DEPARTMENTS

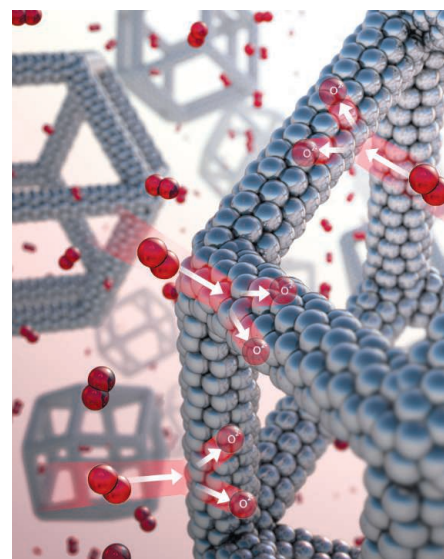
- 1288 This Week in *Science*
1291 Editors' Choice
1292 Science Staff
1378 New Products
1379 Science Careers

RESEARCH ARTICLE

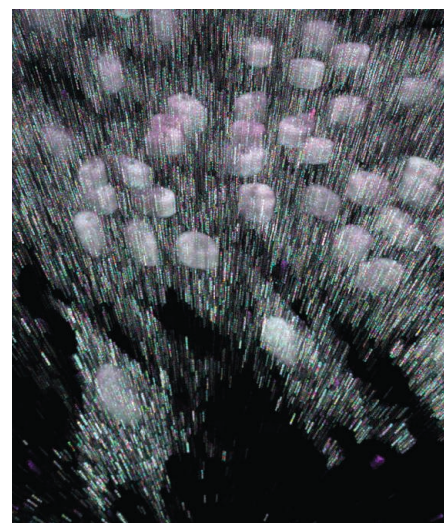
- 1329** Nucleocytoplasmic Shuttling of a GATA Transcription Factor Functions as a Development Timer
H. Cai et al.
The link between chemoattractant gradients, developmental signals, and gene expression in social amoebae is elucidated.
Research Article Summary; for full text: <http://dx.doi.org/10.1126/science.1249531>
>> *Perspective p. 1326*

REPORTS

- 1330** Super-Eddington Mechanical Power of an Accreting Black Hole in M83
R. Soria et al.
A stellar-mass black hole shocks its surroundings with kinetic energy in excess of its predicted radiative output.
>> *Perspective p. 1318*
- 1333** Large-Amplitude Spin Dynamics Driven by a THz Pulse in Resonance with an Electromagnon
T. Kubacka et al.
The electric field of an electromagnetic pulse exerts ultrafast control on the spin dynamics of the multiferroic TbMnO₃.
- 1336** Angular Fluctuations of a Multicomponent Order Describe the Pseudogap of YBa₂Cu₃O_{6+x}
L. E. Hayward et al.
A model reproduces the temperature dependence of charge-order fluctuations in a cuprate superconductor.
- 1339** Highly Crystalline Multimetallic Nanoframes with Three-Dimensional Electrocatalytic Surfaces
C. Chen et al.
Highly active electrocatalysts are created by eroding away all but the edges of platinum-nickel nanocrystals.
>> *Perspective p. 1319*
- 1343** The Source Crater of Martian Shergottite Meteorites
S. C. Werner et al.
Martian meteorites originated from the 3- to 5-million-year-old Mojave impact crater.
- 1347** Iron Fertilization of the Subantarctic Ocean During the Last Ice Age
A. Martínez-García et al.
Nitrogen isotopes in foraminifera show the role of iron fertilization on atmospheric carbon dioxide during the last ice age.
- 1350** From Parasitism to Mutualism: Unexpected Interactions Between a Cuckoo and Its Host
D. Canestrari et al.
The carrion crow *Corvus corone corone* can benefit from parasitism by the great spotted cuckoo *Clamator glandarius*.
- 1353** β -Catenin Activation Regulates Tissue Growth Non-Cell Autonomously in the Hair Stem Cell Niche
E. R. Deschene et al.
Signals generated by mouse hair follicle stem cells generate new hair growth.
- 1357** Activity of Protein Kinase RIPK3 Determines Whether Cells Die by Necroptosis or Apoptosis
K. Newton et al.
A particular protein kinase functions at a critical control point that determines whether—and how—cells die.
>> *Perspective p. 1322*
- 1360** Highly Multiplexed Subcellular RNA Sequencing in Situ
J. H. Lee et al.
Reads of cellular RNA transcripts demonstrate spatial expression differences during simulated wound healing.
- 1363** Structure of the Mitochondrial Translocator Protein in Complex with a Diagnostic Ligand
Ł. Jaremko et al.
A ligand stabilizes a mammalian mitochondrial cholesterol transporter, allowing high-resolution structural analysis.
- 1366** Epistasis and Allele Specificity in the Emergence of a Stable Polymorphism in *Escherichia coli*
J. Plucaín et al.
The emergence of a stable polymorphism in bacteria involved a multistep process including three specific mutations.
- 1370** Humans Can Discriminate More than 1 Trillion Olfactory Stimuli
C. Bushdid et al.
The number of different odor mixtures people can distinguish is several orders of magnitude larger than anticipated.
>> *Science Podcast*
- 1373** Control Profiles of Complex Networks
J. Ruths and D. Ruths
The control profile is a network statistic that may prove useful in approaching the control of complex networks.
>> *Perspective p. 1325*
- 1376** Fossilized Nuclei and Chromosomes Reveal 180 Million Years of Genomic Stasis in Royal Ferns
B. Bomfleur et al.
Fern fossils provide evidence that nuclear shape, size, and chromosomal content have changed little since the Jurassic.



pages 1319 & 1339



page 1360

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There Goes the Neighborhood

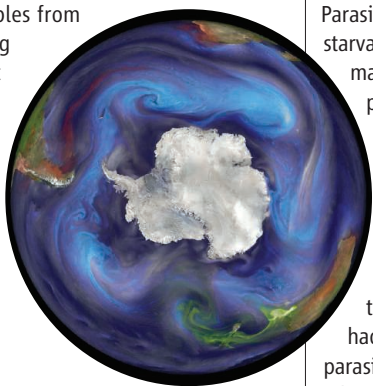
A mass-accreting black hole in steady-state cannot produce more radiative energy than its gravity can counterbalance, achieving what is known as the Eddington limit. However, mass accretion can also be converted into kinetic energy via mechanical outflow. Using x-ray observations, **Soria et al.** (p. 1330, published online 27 February; see the Perspective by **King** and the cover) identified a compact shock-ionized radio/optical nebula in spiral galaxy M83, powered by a black hole, inferred that the black hole emits a spherical wind that exceeds the Eddington limit tenfold and succeeded in estimating its mass in the range of 5 to 15 solar masses. It is possible that rapidly accreting black holes have greater influence on their host galaxy than once appreciated.

Sourcing Martian Meteorites

There are nearly 150 recognized martian meteorites, but where exactly they came from on Mars is not known. **Werner et al.** (p. 1343, published online 6 March) present evidence that the <5 million-year-old Mojave impact crater on Mars is the single ejection site of one type of martian meteorites: the shergottites. The Mojave crater formed on an ancient terrain on Mars, and so the shergottites represent old martian crustal material.

Productive Dustiness

The idea that biological productivity in the surface ocean is limited by a lack of available iron has been widely accepted, but it has been difficult to show that this effect might have operated in the geological past. **Martínez-García et al.** (p. 1347) investigated the isotopic composition of foraminifera-bound nitrogen in samples from an Ocean Drilling Project sediment core and found millennial-scale changes in nitrate consumption correlated with fluxes in the iron burial and productivity proxies over the past 160,000 years. Hence, in the Southern Ocean the biological pump was strengthened when dust fluxes were high, which explains a significant part



Cytologically Informative Fossils>>

Fossilization processes tend to destroy fine-cell structure but, exceptionally, **Bomfleur et al.** (p. 1376) have found examples of fossil ferns from the Jurassic in which subcellular structures, including organelles such as nuclei and chromosomes, are well-preserved. Comparative and quantitative analyses show that these cells closely resemble the fossil nuclei of extant cinnamon ferns, *Osmundastrum cinnamomea*, which indicates that this group of ferns has remained virtually unchanged for 180 million years.

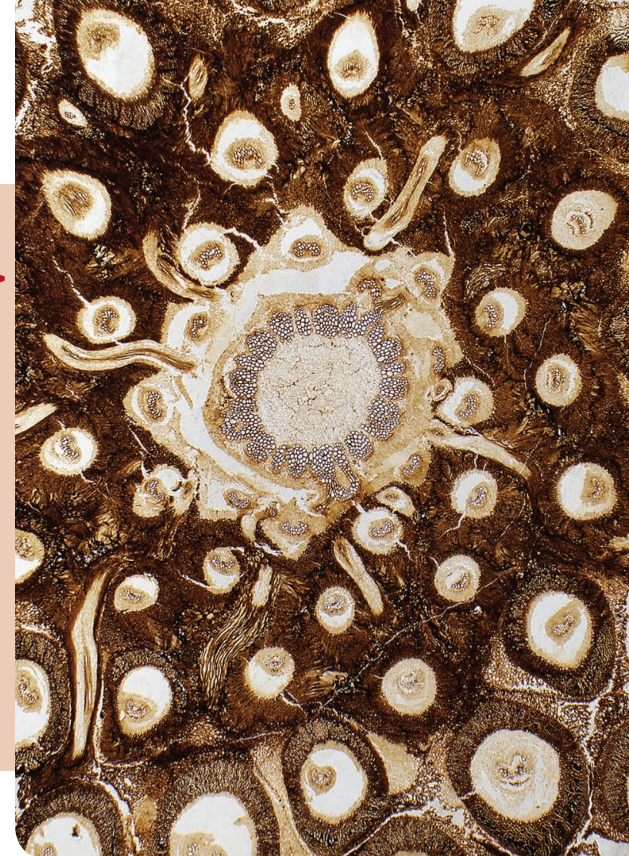
of the difference in atmospheric CO₂ concentrations observed to occur across glacial cycles.

All the Smells of the World

How many odorant stimuli can a normal human being discriminate? During psychophysical tests of odor mixture discrimination, **Bushdid et al.** (p. 1370) were surprised to find that humans can discriminate among more than a trillion different smells. Because the authors reduced the complexity by investigating only mixtures of 10, 20, or 30 components drawn from a collection of 128 odorous molecules, this astonishingly large number is probably the lower limit of the potential number of olfactory stimuli that humans can distinguish.

Predation Favors Parasitism

Parasitism in birds often results in ejection or starvation of the host's nestlings. Consequently, many host bird species have evolved protective behavior such as mobbing and parasite egg rejection. Curiously, some host species show no parasite avoidance behaviors; for example, the crow *Corvus corone corone* tolerates cuckoo chicks among its own brood. In a long-term study, **Canestrari et al.** (p. 1350) found that crow nests containing a cuckoo chick had lower rates of predation because the parasite's chicks secrete a noxious repellent substance. Overall, in years of high predation pressure, the presence of cuckoos improves the crow's breeding success, but when there are fewer predators around, parasitism reduces crow fitness.



Giving Electrocatalysts an Edge

Platinum (Pt) is an excellent catalyst for the oxygen-reduction reaction (ORR) in fuel cells and electrolyzers, but it is too expensive and scarce for widespread deployment, even when dispersed as Pt nanoparticles on carbon electrode supports (Pt/C). Alternatively, **Chen et al.** (p. 1339, published online 27 February; see the Perspective by **Greer**) made highly active ORR catalysts by dissolving away the interior of rhombic dodecahedral PtNi₃ nanocrystals to leave Pt-rich Pt₃Ni edges. These nanoframe catalysts are durable—remaining active after 10,000 rounds of voltage cycling—and are far more active than Pt/C.

Coordinated Hair Growth

Wnt/β-catenin signaling is a key pathway that plays a conserved role in regulating stem cell function during adult tissue regeneration. Using time-lapse imaging of live mice, **Deschene et al.** (p. 1353) show that genetic activation of β-catenin within hair follicle stem cells generates axes of hair growth by coordinated cell divisions and cell movements, even when the normal niches—the dermal papillae—are laser-ablated. Activated β-catenin enhances Wnt ligand secretion, and these ligands can then activate Wnt signaling in adjacent cells that do not have activated β-catenin, indicating how activated stem cells could influence neighboring cells during normal growth and in cancer.

CREDITS (TOP TO BOTTOM): SWEDISH MUSEUM OF NATURAL HISTORY; WILLIAM M. PUTMAN AND ARLINDO M. DA SILVA/NASA GODDARD SPACE FLIGHT CENTER

Additional summaries

HIV Virulence

A major focus of research on HIV is on host responses to infection—understandably, because the virus targets the immune system and because of the interest in vaccine development. In reviewing what little research has been done on viral virulence determinants, **Fraser *et al.*** (p. 1328) present evolutionary explanations for some of the poorly understood phenomena that mark HIV infection, including long-term survivorship, latency, rapid within-host evolution, and inheritability of between-host virulence.

Oscillate to Synchronize

In the social amoeba *Dictyostelium discoideum*, periodic waves of the small-molecule cyclic adenosine monophosphate (cAMP) guide chemotactic migration and cell differentiation. **Cai *et al.*** (p. 1329; see the Perspective by **Wollman**) found that a GATA family transcription factor shuttles between the nucleus and the cytoplasm of the amoeba in response to oscillatory cAMP signals acting through G protein–coupled receptors. Each oscillation generates a transient burst of gene activation, such that gene expression is linked to the number rather than the level of stimulus, which then brings about transcriptional synchrony in populations of cells.

Ultrafast Manipulation

Multiferroic materials commonly show both magnetism and ferroelectricity, such that the electric field can be used to manipulate the magnetic order, and vice versa. **Kubacka *et al.*** (p. 1333, published online 6 March) used a strong terahertz electromagnetic pulse in resonance with an electromagnon—an excitation based on both electric and magnetic ordering—to control the spin dynamics of the multiferroic TbMnO_3 on a sub-picosecond time scale and induce the rotation of the spin-cycloid plane of the material.

The Cuprate Pseudogap

The properties of copper-oxide superconductors are changed by chemical doping, but, if doping is suboptimal, the transition temperature T_c drops. Conversely, the so-called pseudogap, a depression in the density of states around the Fermi level that may or may not be related to superconductivity, gains strength. The cuprate $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$ shows a charge density order that grows as T_c is approached from both low and

high temperatures. **Hayward *et al.*** (p. 1336) have developed a model in which classical fluctuations of a six-component order parameter, encompassing both superconducting and charge orders, reproduce the characteristic concave temperature dependence of the x-ray scattering intensity and thus provide a framework for the understanding of the pseudogap regime.

Life and Cell Death

Trying to protect animals from one form of cell death may lead to death by another. Two protein kinases, known as RIPK1 and RIPK3 promote signaling that leads to cell death by necroptosis. However, **Newton *et al.*** (p. 1357, published online 20 February; see the Perspective by **Zhang and Chan**) found that inhibition of RIPK3 was not always beneficial. Instead, mice expressing a form of RIPK3 with no catalytic activity died from increased apoptotic cell death, but animals lacking the RIPK3 protein entirely, did not die perhaps because RIPK3 restrains apoptosis mediated by caspase-8 by an independent mechanism.

Transcripts Visualized in Situ

Despite advances, current methods for single-cell sequencing are unable to resolve transcript location within the cell, so **Lee *et al.*** (p. 1360, published online 27 February) developed a method of fluorescent in situ RNA sequencing (FISSEQ) that works in vivo to show messenger RNA localization within cells. The method amplifies complementary DNA targets by rolling circle amplification, and then in situ cross-linking locks amplicons to produce ample, highly localized templates for three-dimensional sequencing. The technique was tested in fibroblasts to reveal the differences between individual cells during wound repair.

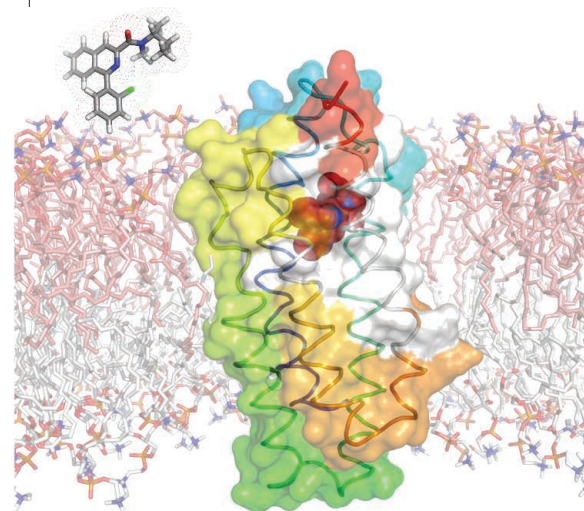
Serial Mutation

Mutations that affect gene function and, ultimately, the phenotype of an organism are grist to the mill of evolution. While examining the genetic basis for a stable polymorphism observed in bacteria during a long-term mutation experiment, **Plucain *et al.*** (p. 1366, published online 6 March) identified three specific, successive mutational events exhibiting synergistic epistatic and frequency-dependent interactions that enabled one lineage to invade

the other and to be maintained. Thus, a series of specific mutations conferred the invasion phenotype and allowed the use of novel resources only when all mutations were present.

Translocation in Injury

The translocator protein TSPO is essential for the import of cholesterol and porphyrins into mitochondria. TSPO expression increases in areas of brain injury and during



neuroinflammation and, thus, has diagnostic and therapeutic implications. **Jaremko *et al.*** (p. 1363) used nuclear magnetic resonance spectroscopy to determine the high-resolution structure of the 18-kilodalton mammalian TSPO with the ligand PK11195, which stabilized the structure and resolved the conformation as a tight bundle of five helices.

Real Network Control

Understanding how complex networks are controlled has implications for a variety of real-world networks, from traffic safety to transcriptional control. **Ruths and Ruths** (p. 1373; see the Perspective by **Onnela**) have developed a theoretical framework for analyzing individual controls within networks based on numbers of sources and sinks for information flow. By this method, the number of controls required by a network can be predicted and direct comparisons for the basis for control across networks of differing size, structure, and function can be made. Although three broad classes of real networks were observed, current, established random models of networks were insufficient to model their control structures.



Marcia McNutt is Editor-in-Chief of *Science*.

Exxon Valdez: 25 Years Later

AFTER I WAS CALLED UPON IN 2010, AS DIRECTOR OF THE U.S. GEOLOGICAL SURVEY, TO HELP WITH well control and flow estimation for the *Deepwater Horizon (DWH)* oil spill, I reviewed what had been learned from the *Exxon Valdez (EV)* oil spill. Although *EV* and *DWH* differed in their spill characteristics and impacts, I suspected that insight gained in the decades since *EV* could help guide how to best invest research funds targeted for *DWH* recovery and future oil spill prevention

The *EV* oil tanker went aground on Bligh Reef on 24 March 1989, releasing approximately 42 million liters of oil into the pristine Prince William Sound, Alaska. The spill covered at least 1990 km of shoreline. Oil cleanup was hampered by the overall inaccessibility of the region and poor preparedness. Only about 14% of the oil was recovered, with some 15% of the remainder coating rocks and contaminating the sandy soil of the sound and Gulf of Alaska beaches.*

A challenge in measuring the recovery of Prince William Sound from the *EV* spill was the lack of a pre-spill baseline for the ecosystem. Therefore, in the early days of the *DWH*, scientists undertook a biological and geological sampling program before any oil hit the shoreline. One recommendation from the experience is that all large marine ecosystems should be routinely surveyed.† Another important lesson from the *EV* spill is to consider recovery time scales in terms of decades or longer. Trace amounts of oil, degraded to an amount expected after 2 weeks of exposure (not decades), can still be found along the Alaska shoreline.‡ Chronic exposure to such oil may still be elevating the mortality and delaying the recovery of sea otters in Prince William Sound. A corollary of this lesson is that natural variability in ecosystems on decadal time scales will confound attempts to monitor recovery from a spill.

Both spills affected the marine ecosystem at multiple tropic levels. Clearly, both impact assessments and response actions should treat all elements of the habitat as a coupled ecosystem. And yet I recall attending a meeting during *DWH* in which a wildlife management official claimed, “Your opinion on the use of dispersants depends on whether you are a bird person or a fish person.” My response was, “Don’t the birds eat the fish?” The cleanup also prompted scientists to ask: “How clean is too clean?” Local Alaskan communities wanted the region returned to as close to its pre-spill state as possible. One unfortunate action was an attempt to “steam clean” the shoreline with high-pressure hot water. The unintended effect was to destroy the resident microbial population that could have facilitated metabolic breakdown of the oil. For this reason, officials were cautious in the amount of cleanup during the *DWH* accident in the fragile Gulf Coast marshes, arguing that trampling marsh grasses in the process of oil recovery would do more harm than good.

Science can not only address oil spills after the fact by guiding actions for response and recovery, but it can also be applied to avoid spills through safety engineering. In a number of industries, accidents have declined through a deliberate consideration of potential failure modes in engineered systems and in their interfaces with humans. Such an analysis is well overdue to prevent oil spills.

— Marcia McNutt

10.1126/science.1253412



*D. A. Wolfe et al., *Environ. Sci. Technol.* **28**, 560A (1994). †J. Lubchenco et al., *Proc. Natl. Acad. Sci. U.S.A.* **109**, 20212 (2012). ‡www.sgmeet.com/osm2014/viewabstract.asp?AbstractID=18111.

EVOLUTION

Skunky or Social

Though we often think of mammals in the order Carnivora as predators, many of these species are themselves subject to high rates of predation and, as such, have evolved a suite of antipredator defenses that tend to sort them into two quite different categories. Such small carnivore species generally tend to be either solitary, often aposematically colored and armed with noxious anal sprays, or social and highly vigilant. Stankowich *et al.* used natural history data, including range overlap with potential predators, body size, and activity patterns, in conjunction with comparative phylogenetic analyses on 181 species of mammals to identify patterns of predation risk that could have contributed to the evolution of these two defensive strategies. They found that species that have evolved noxious sprays as a defense, such as skunks, tend to be nocturnal and subject to predation by other mammals, whereas socially vigilant species, such as mongooses, tend to be diurnal and at risk of predation by birds of prey. These results show how similar ecological contexts can result in the repeated evolution of highly adaptive strategies across species and regions. — SNV

Evolution 10.1111/evo.12356 (2014).

Notch inhibition by a gamma secretase inhibitor increased the fraction of supporting cells that transdifferentiated into hair cells and that the effects of *Notch* were dependent on β -catenin. It is not yet known whether this process can be triggered in older animals. — BJ

Stem Cell Rep. 2, 311 (2014).

ENVIRONMENTAL SCIENCE

Root Down

Owing to their remarkable ability to uptake and translocate compounds using their roots, plants have long been considered a possible means of treating contaminated soils. However, this ability also provides an avenue by which organic contaminants such as pesticides and herbicides may be introduced into the food chain, an issue of great concern in the fields of public and ecosystem health. Collecting data in the field is a robust but slow way to assess exposure pathways, so a more efficient way to do that would be of great value. Limmer and Burken applied a predictive model, based originally on the uptake and translocation of pharmaceuticals and other compounds in human tissues, to plant roots and trace organic contaminants. As expected, hydrophobicity and molecular mass of the organic compounds are two major predictive physiochemical domains of uptake, but a number of other factors, including hydrogen bonding and polar surface area, also are important. Because plant membranes behave in a fashion similar to human membranes such as the blood-brain barrier, plant roots may serve as the primary

protective barrier from exposure to unwanted organic compounds. — NW

Environ. Sci. Technol. Lett. 10.1021/ez400214q (2014).

PHYSICS

Defect Control

Quantum information processing and nanoscale sensing applications rely on the ability to store and manipulate information encoded into quantum states of matter—quantum bits. Some solid-state implementations look to use the spin of electrons in quantum dots as the storage media. The quantum properties of natural or artificial defect centers (nitrogen vacancy or NV centers) in diamond can be optically addressed and manipulated and can be robust even up to room temperature. Likewise, silicon carbide (SiC) has also been found to have defects that exhibit similar properties to those of the diamond NV centers, including long coherence times that persist up to room temperatures, a high degree of optical polarization, and spin-dependent photoluminescence. Klimov *et al.* now demonstrate that the spin properties of defects in SiC can be coherently controlled electrically by applying series of voltage pulses to gates surrounding the defects. The compatibility with Si fabrication techniques makes SiC an ideal candidate for the development of integrated quantum technology platforms using scalable quantum control of electron spins in a dense array. — ISO

Phys. Rev. Lett. 112, 10.1103/PhysRevLett.112.087601 (2014).

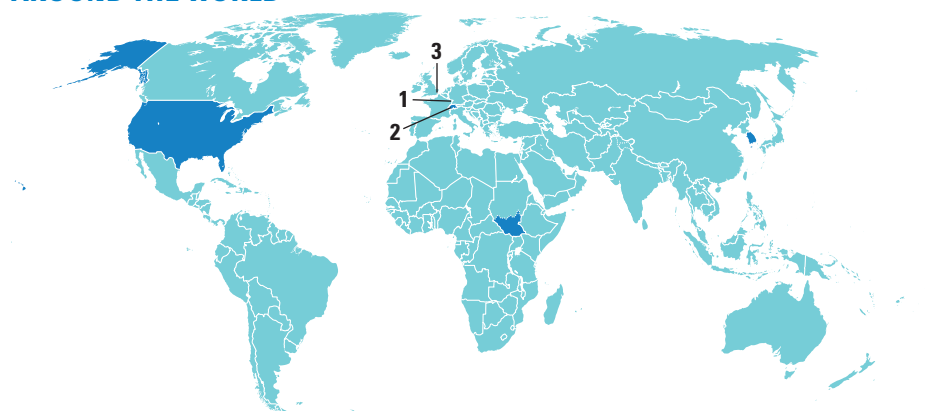
CELL BIOLOGY

Growing Back Hearing?

Hair cells do not normally regenerate in the mammalian ear, and it has been thought that permanent damage to human hair cells in the cochlea inexorably resulted in hearing loss. However, Bramhall *et al.* have found that supporting cells in the cochlea taken from newborn mice can turn into hair cells. In a chemical model of damage, explant cultures were treated with gentamycin, and lineage tracing was done to track cell populations. New hair cells arose at a low level from a subpopulation of supporting cells that expressed the *Lgr5* (leucine-rich repeat-containing G protein-coupled receptor 5) marker, a protein in the *Wnt* signaling pathway. Previous studies had shown that inhibition of the *Notch* signaling pathway can help restore hearing in mice with noise-induced deafness. Here, Bramhall and colleagues found that

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AROUND THE WORLD



Strasbourg, France 1

Parliament Tightens E.U. Data Privacy

The European Parliament has approved stricter rules for the protection of personal data across the European Union—a move that some research organizations fear will undermine public health and medical research.

On 12 March, the members voted overwhelmingly to approve the draft regulation, which updates 1995 rules and includes controversial amendments introduced last fall by Parliament's civil liberties committee. In a



Landslide. The European Parliament voted overwhelmingly to approve tougher data protection rules.

key change, research organizations must get specific consent to use or reuse patients' data unless the research serves a "high public interest ... [and] cannot possibly be carried out otherwise." Supporters call the changes reasonable and necessary, but scientists argue that they are too strict and vague and will threaten important research.

Parliament must still negotiate the reform with the Council of Ministers, which represents the 28 E.U. member states. "We hope

that we can still change things, for the good of biomedical research and for the benefit of patients," says Nathalie Kayadjanian, senior scientific officer for the medical scientific committee of Science Europe, an association of research funders. http://scim.ag/_EUdata

Bern 2

Switzerland to Replace Lost E.U. Funding Opportunities

The Swiss government announced last week that it will step in to help researchers compensate for a funding block from the European Union. After voters agreed in

February to curb mass immigration, Switzerland lost its status as an associated country to Horizon 2020, the European Union's new research funding program, and to the higher education program Erasmus+, both of which run from 2014 through 2020. Individual scientists from Swiss institutions can no longer apply for basic research grants from the European Research Council (ERC) or for Marie Curie scholarships.

To pick up the slack, the Swiss National Science Foundation (SNSF) will offer ERC-like grants, with roughly the same rules, evaluation criteria, and funding calendar. The budget of the temporary fund is not settled yet, says SNSF spokesman Alan Knaus, but will "presumably" come from money Switzerland would have paid the European Union to become an associated country in Horizon 2020.

While the scientific community is "grateful," says Martine Rahier, president of swissuniversities, "a full membership in the Horizon 2020 and ERC programs remains crucial." <http://scim.ag/SwissERC>

London 3

U.K. Report Condemns Europe's GM Crop Policy

Prominent U.K. plant biologists and biotechnologists criticized the European Union's "dysfunctional approval process" for genetically modified (GM) crops in a government-funded report released last week. The research, commissioned by the U.K. Council for Science and Technology, found that the European Union has stifled scientific progress with its regulations and allowed political considerations to influence its risk assessments.

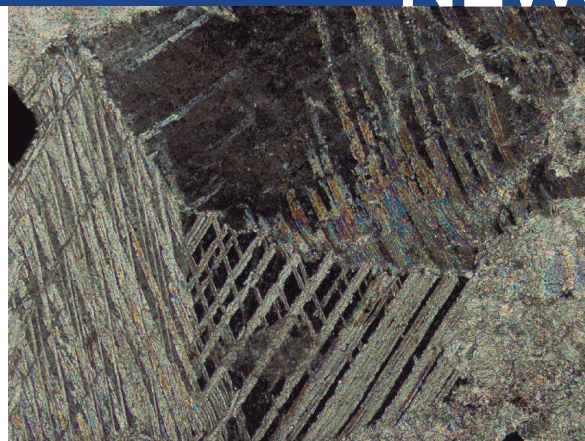


Harsh climate. French protesters uproot GM maize.

The U.K. government already supports GM crops, and the authors make several suggestions for pushing the approval process forward, including a shift of regulatory power away from Brussels and the European Food Safety Authority. They suggest that the United Kingdom create a new authority to vet GM varieties, while the European Union maintains an advisory role on risk and safety.

The report also proposes that GM crops be assessed according to their traits—just like varieties produced via conventional plant breeding—rather than by the process used to produce them. "The regulation of the technology is not proportionate," says Jonathan Jones of the Sainsbury Laboratory in Norwich, U.K., one of the authors. "It's time to remove the red flags."

http://scim.ag/UK_GM



Random Sample

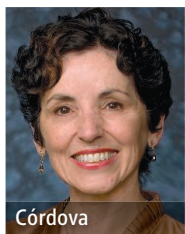
Blarney Myths Go Under the Microscope

Ireland's storied Blarney Stone has received plenty of kisses this week, but it also got some new scientific attention recently, thanks to the chance find of a microscope slide in the Hunterian Museum of the University of Glasgow in Scotland. While digitizing the mostly handwritten catalog of the museum's 40,000 geological slides, intern Becky Smith noticed one entry referring to a slide of the Blarney Stone. The records show that Victorian mineralogist Matthew Forster Heddle acquired the slide sometime between 1850 and 1880, but he doesn't appear to have published anything about it.

Geologists at the museum analyzed the sample, which is cut thin enough to be transparent, to determine its composition. The stone is rumored to be made of Welsh bluestone, the same material used to make the monoliths of Stonehenge, or else of sandstone cleaved from the Stone of Scone, which forms the coronation seat used by the kings and queens of Scotland and Great Britain for hundreds of years. But the new analysis revealed that the landmark is Irish through and through: It is a 330-million-year-old carboniferous limestone typical of that corner of Ireland and contains fragments of fossilized brachiopod shells and bryozoans. Hunterian curator John Faithfull says he has kissed the slide, but the jury is still out on whether he has acquired the legendary "gift of the gab."

NEWSMAKERS

France Córdova Confirmed To Lead NSF



It's been nearly 20 years since **France Córdova** has worked for the U.S. government, but the new director of the National Science Foundation (NSF) says she's never really severed her ties to Washington.

On 12 March, the Senate confirmed the 66-year-old astrophysicist as NSF's 14th director. She replaces Subra Suresh, who left 1 year ago less than halfway through his 6-year term to become president of Carnegie Mellon University.

After spending 3 years as NASA's chief scientist under Daniel Goldin in the mid-1990s, Córdova returned to academia and quickly moved up the administrative ranks. In 2002, she became chancellor of the University of California, Riverside, and in 2007 she began a 5-year stint as president of Purdue University. In 2012, she was named chair of the Board of Regents of the Smithsonian Institution, a post she has relinquished to join NSF. Córdova is expected to take up the reins of the \$7 billion agency in

a few weeks. She says she has kept abreast with issues facing the agency but declined to comment on pending legislation affecting its programs (see p. 1298).

FINDINGS

'Little Foot' Fossil Could Be A Human Ancestor

The age of Little Foot—the most complete skeleton known of an early member of the human lineage—has vexed researchers since his 1997 discovery in a South African cave. But a new study suggests he may be old enough to be a direct ancestor of today's humans.



Little Foot's discoverers, paleoanthropologist Ron Clarke of the University of the Witwatersrand in Johannesburg, South Africa, and colleagues, first pegged him to be about 3.3 million years old. But other teams arrived at about 2.2 million, meaning Little Foot couldn't be ancestral to the first (2.5-million-year-old) *Homo* fossils. After a more detailed analysis of the cave rocks, Clarke's team now argues that 2.2 million may be a good estimate for the so-called flowstones that formed as water deposited minerals in the space around the skeleton. But Little Foot himself is at least 3 million years old, they claim in a paper in press at the *Journal of Human Evolution*. Some are skeptical, but if correct, the results put South Africa back in the running as a possible home of the *Homo* line. http://scim.ag/_littlefoot

THEY SAID IT

"This has been at once the best and worst job I've ever had."

—David Wright, director of the U.S. Office of Research Integrity, in a resignation letter describing how the "dysfunctional" federal bureaucracy drove him to quit after 2 years. <http://scim.ag/WrightORI>

CREDITS: (TOP LEFT TO RIGHT) © TERRY MATHEWS/ALAMY; UNIVERSITY OF GLASGOW'S HUNTERIAN MUSEUM; (BOTTOM LEFT TO RIGHT) MARK SIMONS/PURDUE UNIVERSITY; © RON CLARKE



Long, cold stare. From its South Pole perch, BICEP2 (foreground) peers at the sky.

COSMOLOGY

First Wrinkles in Spacetime Confirm Cosmic Inflation

Few cosmologists were surprised on Monday, when observers announced that they had spotted traces of gravitational waves—undulations in the fabric of space and time—rippling through the infant universe. Rumors of the discovery had circulated for days. Yet, the observation electrified scientists the world over. That's because, if it holds up, it clinches the idea that in its first sliver of a second, the cosmos expanded like a gargantuan balloon in a faster-than-light growth spurt known as inflation—a wild idea proposed more than 30 years ago. It also shows for the first time that gravity must follow the same rules of quantum mechanics that other forces such as electromagnetism do. Forging a quantum theory of gravity may be the grandest goal in theoretical physics.

Some cosmologists say the discovery is the biggest in their lives. “Never has the boundary of human understanding been pushed back so far,” says Max Tegmark of the Massachusetts Institute of Technology (MIT) in Cambridge, who was not involved in the work. Researchers had good evidence of how the first atomic nuclei formed a second after the big bang. But now they have probed the first 10^{-32} seconds, says Marc Kamionkowski, a cosmologist at Johns Hopkins University in Baltimore, Maryland. “It’s not every day that you wake up and find out what happened one trillionth of a trillionth

of a trillionth of a second after the big bang.”

The discovery comes from a study of the big bang’s afterglow, the cosmic microwave background (CMB). Cosmologists with the Background Imaging of Cosmic Extragalactic Polarization (BICEP), a small but sophisticated telescope at the South Pole, mapped how the arrowlike polarization of those microwaves varies from place to place across the sky. In data taken from January

“It’s not every day that you wake up and find out what happened one trillionth of a trillionth of a second after the big bang.”

—MARC KAMIONKOWSKI,
JOHNS HOPKINS UNIVERSITY

2010 to December 2012, they found faint pinwheel-like swirls called B modes. “We believe that gravitational waves could be the only way to introduce this B-mode pattern,” says John Kovac, a cosmologist at Harvard University and one of the four

principal investigators of BICEP. The results were announced in a talk at the Harvard-Smithsonian Center for Astrophysics in Cambridge.

Many cosmologists also consider B modes the smoking gun for inflation. According to the standard model of cosmology, when the universe sprang into existence it contained one thing: a quantum field, similar to an electric field, made up of particles called inflatons. That field blew up spacetime so that within 10^{-32} seconds the cosmos doubled and redoubled its size 60 times. In the process, it pulled itself “flat” like a bed sheet snapping taut and evened out in temperature. Inflation stopped as the inflatons decayed into other particles, ultimately including photons, electrons, and quarks. That inflationary scenario was invented in 1980 by Alan Guth, a cosmologist at MIT.

The inflaton field roiled with tiny quantum fluctuations. Inflation magnified those fluctuations to enormous size, seeding variations in the density of energy and matter that eventually grew into galaxies. The fluctuations also created part-in-100,000 variations in the temperature of the CMB across the sky. By measuring the statistical distribution of hot and cold spots of different sizes, researchers have determined the content of the universe in terms of ordinary matter, mysterious dark matter whose gravity binds the galaxies, and weird space-stretching dark energy (*Science*, 29 March 2013, p. 1513).

That much of the evolution of the universe has been traced, and it all appears to be consistent with the idea of inflation. But with the new results, researchers have gone a big step further and tested a particular prediction of inflation. Thanks to quantum mechanics, not only did the stuff inside the infant universe fluctuate—so did spacetime itself. Or so it must have if spacetime and gravity are quantum mechanical. Inflation stretched that jittering into gravitational waves billions of light-years in wavelength that left their own imprint on the CMB. Whereas the density variations caused a simple sloshing of matter and energy from more dense spots to the less dense ones, gravitational waves stirred up a more complex twisting motion called “tensor modes.” Only that type of motion can give rise to B modes, says Uros Seljak, a cosmologist at the University of California, Berkeley.

Spotting those modes wasn’t easy.

CREDIT: STEFFEN RICHTER/HARVARD UNIVERSITY

The B modes are only 1% as strong as the already-faint temperature variations. To see them, the BICEP team deployed BICEP2, a 26-centimeter telescope with 500 exquisitely sensitive microwave detectors called bolometers, each cooled to within a fraction of a degree of absolute zero. Researchers got a little help from nature, as the B-mode signal appears about 20 times stronger than many cosmologists had expected.

BICEP scooped a gaggle of other experiments, including the European Space Agency's Planck spacecraft, which took data from 2009 until last year and is expected to present polarization data soon. Ironically, Kovac says, BICEP owes its success in part to detectors made by Jamie Bock and colleagues at the California Institute of Technology in Pasadena, who also developed detectors for Planck. Suzanne Staggs, a cosmologist at Princeton University who works on the Atacama B-mode Search in Chile, says she was shocked when she heard of BICEP's success. "The more I think about this, the more excited I am because the signal is so big," she says.

In particular, the big signal suggests that cosmologists may soon be able to test the idea of inflation in earnest. If nothing else, many researchers say, it should silence doubters of the faster-than-light stretching. That's because alternative theories do not produce B modes, says Scott Dodelson, a cosmologist at Fermi National Accelerator Laboratory (Fermilab) in Batavia, Illinois. "All of the alternatives that have been proposed are dead," he says. "This is a done deal."

Now cosmologists hope to probe the characteristics of the inflaton field—particularly how the field interacted with itself to give itself energy. Cosmologists think of the field like a marble on a hillside, with height denoting the field's energy and horizontal position denoting its amplitude. The field started somewhere up on the hill and rolled down toward zero energy and amplitude. BICEP's result reveals the marble's initial height, Dodelson says, which is equivalent to the energy density of the universe during inflation—3 trillion times any energy achieved with a particle accelerator. Cosmologists' next big goal is to determine the shape of the energy landscape, or "potential."

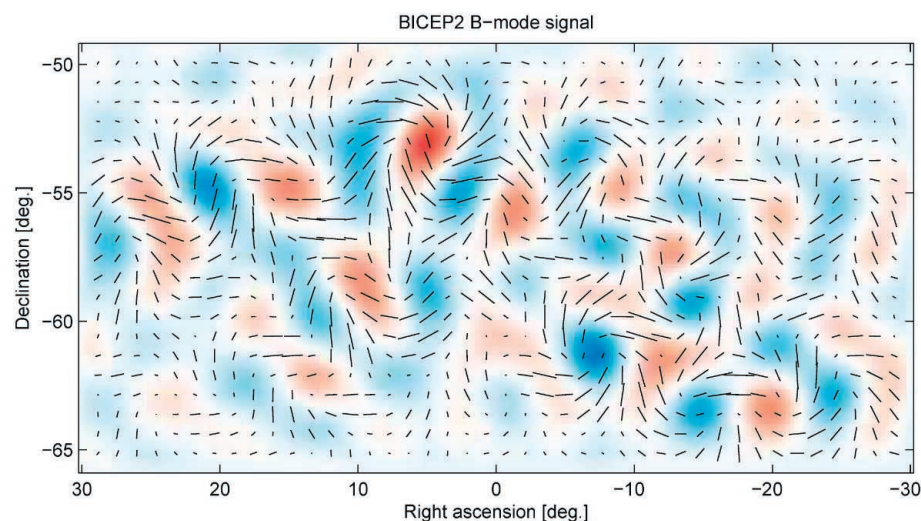
Many cosmologists say that the signal strength reported by BICEP jibes nicely with a model of that landscape proposed in 1982 by Andrei Linde of Stanford University in California, which is a parabola. "Suddenly

this very simple model works very well," says MIT's Tegmark.

A few cosmologists remain inflation holdouts, however. Paul Steinhardt of Princeton University has called inflation "contrived" and says the BICEP results just make it worse. BICEP's B-mode signal implies that the tensor churning in the early universe is twice as strong as the upper limit inferred from Planck's temperature measurements, he says. To make those two observations mesh, the spectrum of shorter and longer quantum fluctuations in the infant universe must have been a lot more complicated than standard theory assumes, he argues: "That's not good for inflation."

larger and smaller B modes should reveal the shape of the inflaton potential, says Berkeley's Seljak. The BICEP team has measured the modes in a patch of sky measuring 15° by 60° and has observed B modes that make pinwheels about a degree wide. Primordial gravitational waves should also produce B modes stretching about 10° across. Spotting those bigger B modes would most likely require another more sensitive spacecraft, which, like Planck, could map the whole sky. "The community will probably make the case for another satellite mission to measure polarization," Seljak says. "That's where I expect we will go."

Perhaps most tantalizing, such a mission



Twisted. Pinwheel-like swirls in BICEP's polarization map of the microwave background are the prized B modes.

Steinhardt acknowledges that the discovery rules out his own noninflationary models—in which big bangs occur over and over again within a much older spacetime. But when the dust settles, he predicts, theorists may still find themselves searching for an alternative to inflation.

Only more observations can settle the issue, scientists say. First off, researchers need to confirm the BICEP result, which may happen fairly quickly if the signal is as large as reported. Beyond that, to trace the inflaton's energy landscape, observers must measure the statistical distribution of the swirling B modes in exactly the same way they measured the statistical distribution of the hot and cold spots. Researchers break the hot and cold spots down into overlapping spots of bigger and smaller sizes on the sky, and the spectrum of different size spots encodes the recipe for the universe.

In much the same way, the spectrum of

might finally enable physicists to test theories that attempt to meld quantum mechanics and Einstein's general theory of relativity, which says that gravity arises when mass and energy bend spacetime. The BICEP result proves that gravity must be quantum mechanical, says Fermilab's Dodelson, as B modes originate from quantum fluctuations in spacetime itself.

Moreover, Dodelson says, theories of quantum gravity, such as string theory, predict modifications to the shape of the inflaton energy landscape. So if that landscape can be measured precisely, he suggests, physicists might finally put string theory—long mocked as an untestable "theory of anything"—to a concrete test.

Even if that dream doesn't come true, the observation of primordial gravitational waves has shaken up cosmology almost as much as the waves did the fledgling universe.

—ADRIAN CHO AND YUDHIJIT BHATTACHARJEE

U.S. SCIENCE POLICY

Hints of Detente Between NSF and Republicans

“Truce” may be too strong a word. But congressional Republicans and the National Science Foundation (NSF) appear to be creeping toward common ground in their yearlong fight over how the agency manages its \$7 billion research portfolio.

Last week, a panel of the House of Representatives’ science committee endorsed controversial legislation (H.R. 4186) reshaping programs at NSF, the country’s largest supporter of academic basic research in the physical and computing sciences and engineering. Some of the provisions in the bill, now awaiting action by the full committee, are markedly less radical than earlier drafts that the scientific community regarded as a direct attack on NSF’s vaunted system of peer review (*Science*, 3 May 2013, p. 534). At the same

time, NSF officials announced that they will be taking a much more active role in deciding how funded grants are explained to the public.

The timing is not coincidental. Republican lawmakers have ridiculed specific NSF awards and questioned whether they serve the national interest. Early drafts of what is now called the Frontiers in Innovation, Research, Science, and Technology (FIRST) Act contained language that, in effect, would have required NSF to certify that its bread-and-butter grants would generate relatively short-term payoffs. The suggested criteria included bolstering the nation’s economy, security, and scientific workforce. NSF also would have had to identify the staffer making each award.

The bill’s backers intended the provisions to promote accountability. But critics said they were setting NSF up for failure by asking its officials to do the impossible. In contrast, the bill marked up last week talks in more general terms about research

taxpayer money that would have been better spent on higher priorities.” (Smith has declined to discuss the bill with *Science*.)

Not surprisingly, the panel’s Democrats take issue with Smith’s view. Their version of the reauthorization bill (H.R.

4159; see sidebar) asks Congress to declare that “the Foundation’s process for selecting proposals for funding ... remains the gold standard for the world.”

A few hours after the subcommittee voted, acting NSF Director Cora Marrett acknowledged the need for “improved transparency and accountability.” In her first public comments on the efforts of an internal working group formed last fall to address these issues, Marrett told *Science* she has reminded program managers that the titles

and abstracts of winning proposals “belong to NSF” and that the staff must do a better job of explaining why the agency is funding each project. If necessary, she added, that could mean rewriting titles and abstracts submitted by investigators before the information is posted on NSF’s website and read by the public—and by curious lawmakers.

Marrett did not directly connect the changes with the debate over the FIRST Act. “I haven’t even looked at the most recent version of FIRST to see what it says about this,” Marrett said. “We just wanted to be sure we had processes in place that we could stand behind. If that results in meeting some congressional requirement, then fine.”



Common ground? Acting NSF Director Cora Marrett’s push to improve grant abstracts addresses concerns raised by Representative Lamar Smith (R–TX).



“having the potential” to do good things for society and doesn’t require the agency to shine a spotlight on individuals.

Representative Lamar Smith (R–TX), chair of the Committee on Science, Space, and Technology and the bill’s primary author, still believes that Congress needs to keep a close eye on NSF, one of several agencies under the committee’s jurisdiction. Provisions relating to how NSF manages grants, for example, fall under a section labeled “Greater accountability in Federal funding for research.” Speaking before the bill was marked up on 13 March, Smith reiterated his belief that “[i]n recent years, the NSF has funded a number of questionable research grants—using up

amounts are insufficient to meet the challenges NSF faces.

The Frontiers in Innovation, Research, Science, and Technology bill also breaks with the two prior authorization bills (known as the America COMPETES Act) by spelling out funding levels for each of NSF’s six research directorates, including a 40% cut in current spending for the social and behavioral sciences. Democrats fought successfully at last week’s markup to restore half of that cut (a moot point for 2014), and criticized such directorate-level funding as micromanagement. But Republicans say the approach allows them to express support for more research within certain disciplines they believe make the largest potential contributions to society.

—JDM

Dueling Visions for Agency

The Democratic version of a controversial Republican bill that would reshape National Science Foundation (NSF) programs hews closer to the traditional function of such reauthorization bills, including a longer time frame and “aspirational” budgets. The bill (H.R. 4159) calls for 5% annual budget increases through 2019 at NSF and two other science agencies and codifies several existing initiatives.

In marked contrast, the Republican bill would expire in 2015. Although its proposed 1.5% increase for that year is larger than the president’s request for a 1.2% boost, Democrats say that both

CREDITS: (LEFT TO RIGHT) FLICKR/NASA; HOUSE OF REPRESENTATIVES COMMITTEE ON SCIENCE, SPACE, AND TECHNOLOGY

The added scrutiny will apply only to proposals that have already passed muster with review panels and are recommended for funding, she emphasized. At that point, however, senior managers will be expected to blow the whistle if they think the abstract doesn't reflect both what a scientist wants to do and why that work is worth funding. Division directors "might possibly" even reject a proposal at the last minute based on an adverse reaction to an abstract, she added.

Marrett doesn't think the new approach will mean any more work for busy staffers. "First of all, it's not that most of the recommended awards are obscure," she noted. "Second, it's something we're already supposed to have been doing. And third, it's not that hard to rewrite the abstract. Sometimes all it takes is putting the nontechnical description of the project ahead of the technical description."

The increased transparency, she said, won't affect the two criteria that NSF uses to judge the quality of each proposal—

intellectual merit and broader impacts. Nor will scientists need to describe short-term, practical spinoffs if none are anticipated, she said, giving as an example an astronomy grant designed to "advance our understanding of the physical world. ... It's not about identifying the consequences of the research for society."

It's not clear how NSF's changes might influence debate over the FIRST bill, which is expected to be taken up next month by the full science committee. No Democrat signaled support for the full bill during a voice vote at last week's subcommittee, although the panel accepted nine minor amendments from the minority side. And Democrats say there is still plenty in the bill that should concern researchers, including provisions to clamp down on scientific misconduct and to limit to five the number of publications cited by grant applicants. "The collective message sent by these provisions is that we don't trust scientists," said Representative Eddie Bernice Johnson (D-TX), the full committee's top Democrat,

at the markup. "Is this really the message the Science Committee wants to be sending?"

Still, Representative Dan Lipinski (D-IL), the top Democrat on the subcommittee, thinks that "the accountability provisions are tremendously better now." Speaking with *Science* after the markup, Lipinski said, "I thought what they had before would have been very harmful to the entire peer-review process at NSF. I don't think NSF will be happy because it will be a little bit more work. But it will not hamper or change the way NSF does its peer-review process."

Science lobbyists are hoping that a similar bill being drafted by the Democrat-controlled Senate Commerce, Science, and Transportation Committee will be more to their liking. Even so, Congress is not required to complete work on any reauthorization bill this year. For now, however, the negotiations appear to be taking place in an atmosphere that has warmed slightly since the war of words began 1 year ago.

—JEFFREY MERVIS

STEM CELLS

Irreproducibility Dogs New Reprogramming Method

TOKYO—Less than 2 months ago, Haruko Obokata suddenly became a media sensation in Japan. The 30-year-old stem cell scientist was widely celebrated when she and colleagues published two papers in *Nature* describing a new and surprising way of creating prized stem cells: by simply subjecting mature cells to stress.

Obokata is still in the spotlight, but the script has changed. Allegations that the papers contain images recycled from Obokata's Ph.D. thesis, among other problems, have fed doubts about the claims. Although dozens of researchers have tried, no one outside the team has reported being able to make the cells, called stimulus-triggered acquisition of pluripotency (STAP) cells, with Obokata's methods. And *Science* has learned that aside from her group, only one of the other labs involved in the papers says it is able to generate STAP cells. One group has tried without success. Others had not even attempted to reproduce the technique until the doubts emerged.

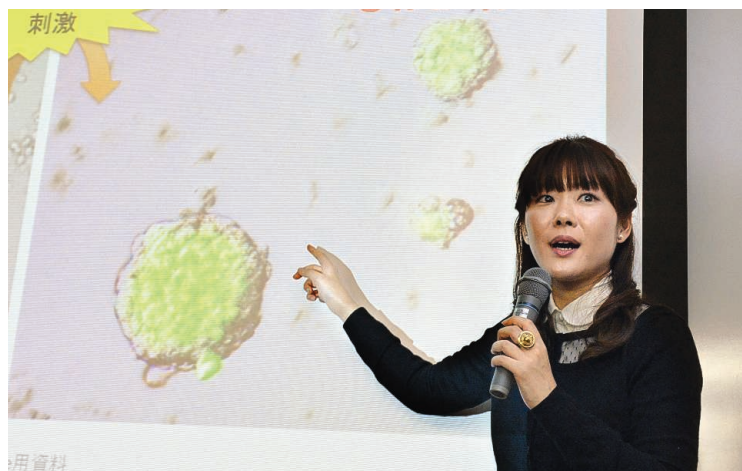
It would not be unusual for the lead author to perform most of the work for the papers, says Martin Pera, a stem cell scientist at University of Melbourne in Australia. But given how revolutionary—

their possible role in regenerative medicine, required extracting them from embryos or forcing adult cells to overexpress certain genes. But in an article and a letter online in *Nature* on 29 January, Obokata and her colleagues at the RIKEN Center for Developmental Biology (RIKEN CDB) in Kobe, Japan, and at other institutions in Japan and the United States described an astonishingly simple alternative. All it took to make pluripotent stem cells, they reported, was briefly bathing blood cells from newborn mice in a mildly acidic solution and then tweaking the culture conditions.

Within days, questions about images in the two papers started appearing on blogs and the PubPeer website. RIKEN CDB's

Tokyo-based parent organization, RIKEN, launched an investigation on 13 February. New allegations about these and previous Obokata papers continued to emerge.

On 14 March, the investigative committee



Media darling. Haruko Obokata explained her radically new method for making stem cells to a packed press conference in January.

and straightforward—the technique was supposed to be, he says, "the uncertainty concerning reproducibility is puzzling."

Earlier methods for deriving pluripotent stem cells, the all-purpose cells valued for

chair and other RIKEN officials announced the interim results at a standing-room-only press conference in Tokyo. RIKEN president and Nobel laureate Ryoji Noyori opened the event with a deep bow. “I apologize for the great trouble and concerns caused to so many in society by the STAP papers published in *Nature* by RIKEN researchers,” he said.

The investigating committee concluded that there had been “inappropriate handling of data” for two of the items under investigation, but these were “not judged to constitute research misconduct,” according to a RIKEN press release. But the officials emphasized that they are relying on the scientific community to ultimately decide whether STAP cells can be derived following the method Obokata and her colleagues described. “The mission of the investigating committee is to determine whether or not there has been misconduct; whether STAP cells exist is something for the scientific community to determine,” said committee chair Shunsuke Ishii, a RIKEN molecular geneticist, at the press conference, which lasted a marathon 4 hours. He added that to the best of their knowledge, no outside group has reported success in generating STAP cells.

Among the authors, only Charles Vacanti, an anesthesiologist and tissue engineering specialist based at Brigham and Women’s Hospital in Boston, says his lab can make STAP cells. Obokata worked under Vacanti for several years, starting in 2008, and developed the STAP technique based on some of his earlier work. Another co-author, Teruhiko Wakayama, who moved in 2012 from RIKEN CDB to the University of Yamanashi, Kofu, confirmed in an e-mail to *Science* that he had made STAP cells while working alongside Obokata at RIKEN but has not been able to reproduce them at his new lab.

Two other co-authors, Hitoshi Niwa and Yoshiaki Sasai of RIKEN CDB, had not attempted to generate the cells independently in their labs before the papers were published. “I’m confused,” says Hans Schöler, a stem cell researcher at the Max Planck Institute for Molecular Biomedicine

in Münster, Germany. Niwa and Sasai are highly regarded scientists, he says, and their names as authors lent the papers substantial credibility. RIKEN CDB Director Masatoshi Takeichi said at the press conference that Niwa’s lab is now working to reproduce the results independently of Obokata.

Wakayama’s own doubts have grown. A stem cell biologist known for work cloning

“I want to believe ...
[but] I no longer know to
what extent Obokata’s
story is the truth.”

—TERUHIKO WAKAYAMA,
UNIVERSITY OF YAMANASHI, KOFU

mice, says he was brought onto the team to produce the chimeric mice described in the paper. He used cells provided by Obokata, he says. “I want to believe” in STAP cells, Wakayama says. However, he says, the photos that convinced him that the cells were real are now believed to have come from a completely different experiment reported in Obokata’s doctoral dissertation. “I do not think this is a simple mistake,” Wakayama

the investigating committee had resolved two of six specific issues in the papers. One problematic image is an artifact of image compression and “not falsification or improper conduct,” the interim report states. But the claims that figures in the article were used in Obokata’s thesis seem to be on target. Obokata and one of her co-authors told the committee that these figures were used by mistake. Ishii emphasized that this issue is still under investigation and any judgments about misconduct will come after further deliberation.

In a written statement released to the press, Obokata, Niwa, and Sasai apologized for the confusion resulting from the uncertainties and inaccuracies in the papers and wrote: “We are contacting other co-authors regarding the possibility of retracting these papers.”

Vacanti is reluctant. “In the absence of compelling evidence that the data presented is incorrect, I do not believe that the manuscripts should be retracted,” he wrote in a statement.

A *Nature* spokesperson told *Science* by e-mail that the journal’s own investigation “is still in progress.” Not all the authors would necessarily have to agree to a retraction, the spokesperson wrote.

The *Nature* papers are not the only Obokata publications under fire. Allegations surfaced last week that large portions of one

chapter of her doctoral thesis, submitted to Waseda University in 2011, appear to have been copied from a National Institutes of Health website and that footnotes to other chapters were copied and pasted from other publications. Local media have reported that Obokata said she intends to withdraw the dissertation. A Waseda public relations official confirmed that a faculty member had received such an e-mail but said that the university, which is investigating, has not

received a formal request from Obokata. If the thesis is retracted, Obokata will lose her doctoral degree.

The glare of the spotlight is not always comfortable.

—DENNIS NORMILE AND GRETCHEN VOGEL



Apologetic. RIKEN leaders present the interim results of an investigation into the *Nature* stem cell papers on 14 March. The committee found “inappropriate handling of data.”

says, adding, “I no longer know to what extent Obokata’s story is the truth.” On 10 March, Wakayama called for the papers to be retracted, at least temporarily, until errors are corrected and results confirmed.

At the press conference, Ishii said that

RESTORATION ECOLOGY

U.S. and Mexico Unleash a Flood Into Colorado Delta

When the influential American conservationist Aldo Leopold canoed down the Colorado River delta to the Gulf of California in 1922, he was awestruck by endless wetlands teeming with wildlife. Leopold vowed to never return, fearing that the wilderness wouldn't live up to his memories. He was right: After the U.S. government repeatedly dammed the river over the next few decades, the water slowed to a trickle and much of the lush habitat became a salt-caked wasteland. Now, however, the delta is about to get a deep drink of water, which should stir new life. "It's a cool experiment," says ecologist N. LeRoy Poff of Colorado State University, Fort Collins. "That little bit of water could be the heartbeat of the ecosystem."

On 23 March, the floodgates of the Morelos Dam near Yuma, Arizona, will open for nearly 2 months, sending water down the lowermost Colorado River for the first time in years. It's just 0.7% of the 18.5 billion cubic meters that once reached the gulf each year, mostly as spring floods. Still, researchers are eager to study what happens. "I'll be there at eight in the morning, to see the first water go through," says Karl Flessa of the University of Arizona in Tucson and co-chief scientist of the experiment. Follow-up research will evaluate the responses of the riverbed, soil, and native vegetation, and perhaps pave the way for future floods.

Right now, almost all of the river channel is bone dry. Mexico uses its 10% allotment of Colorado River water for agriculture and cities. Just 3% of the original extent of cottonwood and willow trees remains. In a few places in the delta, treated wastewater or agricultural runoff sustains wetlands, such as the Ciénega de Santa Clara in Mexico (see map), which boasts 261 species of birds. Elsewhere, the Sonoran Institute of Tucson, Arizona, and other nonprofit groups have been restoring habitat by planting native trees and removing invasive species. The riparian ecosystem is resilient, and adding a bit of water can significantly improve the habitat.

The new water comes thanks to a 2012 amendment to a 1944 water treaty. The main intent of the amendment is to provide water managers with more flexibility, by allowing Mexico to store water far upstream of the border, in Nevada's massive Lake Mead, for example. The deal also calls for next week's experimental flood to help revitalize riverside vegetation and create new sandbars, long-

standing goals of environmental groups that participated in the negotiations.

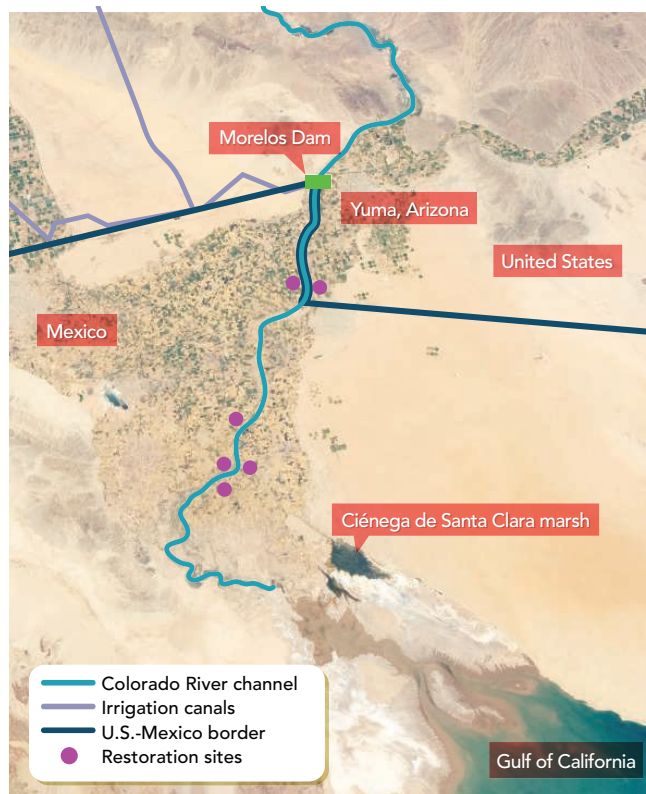
Last May, researchers with universities, government agencies, and nonprofits started to plan the experimental flood. Timing is crucial. The water will arrive while cottonwoods and willows are releasing hundreds of thousands of seeds. The pulse of water should create new sandbars—ideal, moist habitat for seedlings—and also flush salts from the soil, which in places is saltier than the ocean. As floodwater soaks into the ground, it will recharge ground water, perhaps putting it within the reach of the shallow roots of native species. Some of the water will be funneled through a network of agricultural canals, in order to reach existing restoration sites efficiently.

Even during the maximum flow of 120 cubic meters per second, which will last 3 days, the river will probably be no more than hip-deep in places. Larger floods reached the delta in the 1990s, when upstream reservoirs were filled to capacity and had to release water. But this time, researchers are prepared to understand the impact in detail. About 2 dozen scientists, plus graduate students and volunteers, have spent the past several weeks studying the river channel. The U.S. Geological Survey and others have established 22 transects, documenting vegetation, sampling soil, and flying LiDAR surveys for high-resolution topography. The Autonomous University of Baja California, Mexicali, will monitor ground water with shallow wells and hydrologists will track how far the floodwater travels.

After the flooding ends on 18 May, researchers will return to their transects to measure seed germination, and again in late September to look at seedling survival. As the trees grow, researchers will be

able to study emerging forests with aerial photographs or satellite images. "This and subsequent pulse floods will go a long way toward enabling the native species to recover," predicts ecologist David Merritt of the U.S. Forest Service in Fort Collins.

Smaller flows will continue even after May. That's because a coalition of six environmental groups has raised about



Thinking big. An experimental flood released from Morelos Dam will aid restoration of riverside vegetation, even if unlikely to reach the gulf.

\$7.5 million to plant trees and start buying up to 65 million cubic meters of water from Mexican farmers, so it will reach the riverside vegetation rather than be used for agriculture. "As supply gets tighter and tighter, if we want to have water for the environment, we need to secure it legally," says Osvel Hinojosa-Huerta of Pronatura Noroeste, an environmental group in Ensenada, Mexico.

Researchers plan to issue an interim report on the experiment by 2016, when negotiators will consider the next amendment to the treaty. Unlike Leopold, they are hoping for many return trips to the delta.

—ERIK STOKSTAD



A War Within a War

Fighting a major polio outbreak in the midst of Syria's bitter civil war is a test of commitment—and diplomacy

ZA'ATARI REFUGEE CAMP AND AMMAN— Za'atari, sometimes called Jordan's fourth largest city, was thrown together in the desert in a few months' time in 2012. This sprawling, dusty expanse of low-slung tents and prefab housing, 15 kilometers from the Syrian border, has 11 clinics, two schools, and a police station. On its bustling main thoroughfare, dubbed the Champs-Élysées, you can buy fresh fruit and vegetables, mobile phones, electric heaters, canned goods, and cigarettes; bright pink wedding dresses are on display, for sale or rent. Za'atari is now home to at least 100,000 refugees who have fled Syria's brutal civil war, more than half of them children.

Some children arrive sick and severely malnourished. Many have witnessed horrors in the course of the war, which has killed an estimated 140,000 people and displaced

8 million or 9 million. Ripped from their homes and routines, some have seen close family members taken, shot, or killed either in their towns or on the long, dangerous walk to the border. Crammed into tents or prefab trailers with their extended families, with no running water and hand-dug latrines that overflow with the rain, these children are at risk for diarrhea, respiratory disease, hepatitis A, and measles. Scabies and lice are huge problems.

And now they face the threat of polio, which erupted in Syria last October after a 15-year absence and has already paralyzed at least 37 kids—exact numbers are contentious—mostly in the opposition-held north.

"It's an emergency within an emergency," says Australian polio expert Chris Maher—not just for Syria, where millions of kids are at risk, but for surrounding countries as well, including Jordan, Lebanon, and Iraq, as ref-

ugees pour across their borders. And it's another blow to the Global Polio Eradication Initiative (GPEI), a partnership of international agencies that after 25 years has managed to chase the virus out of all but three endemic countries—Pakistan, Afghanistan, and Nigeria—but last year was hit by new outbreaks in the Horn of Africa and now Syria.

That's why Maher, who is 53 and has been fighting polio for more than 20 years, is in nearby Amman leading the biggest polio outbreak response ever attempted in the Middle East. It aims to vaccinate 23 million kids in seven countries, including war-torn Syria, repeatedly over 6 to 8 months and maybe longer. Doing so will involve tens of thousands of vaccinators delivering almost 100 million doses of vaccine. "Anything less and the virus will come and bite you on the ass," says Maher, a senior adviser on polio

CREDIT: SALAH MALKAWI

Bitter medicine. A young Syrian refugee is vaccinated against polio at Za'atari camp in Jordan.

at the World Health Organization (WHO) in Geneva, Switzerland.

The current crisis is “one of the most challenging and visible outbreaks the Global Polio Eradication Initiative has tackled since its launch 25 years ago,” wrote Bruce Aylward, a Canadian epidemiologist and physician who leads GPEI from Geneva, and Ala Alwan, WHO's regional director for the Eastern Mediterranean, in a recent article in *The Lancet*.

Tens of thousands of children are trapped in besieged areas, cut off from any humanitarian intervention, including polio drops. The millions of people on the move, both in Syria and the surrounding countries, are off the map and often out of reach of vaccinators. Fine-grained population mapping, precision vaccination campaigns, and monitoring—the backbone of the eradication initiative—are out of the question.

WHO and other U.N. agencies are walking the finest of lines in Syria, bound by the rules of the Assad regime but determined to vaccinate children in areas where the regime cannot go. Critics have blasted WHO for colluding with the government, vastly underestimating the size of the outbreak, and generally failing the children in the opposition-held north.

So far the response has been far from perfect. “We’ve made millions of mistakes,” Maher says. But still, he adds, “in polio we are now reaching more people in a shorter period of time with an intervention than anyone else has succeeded in doing throughout the whole Syrian crisis”—some 3 million kids so far, each vaccinated multiple times. “And if we can create access for a polio campaign, we can create access for other humanitarian interventions.”

Collapse

On an unusually warm day in January—the snow has just melted—Mohammad Mansour greets me and a translator outside his trailer and invites us in for tea. Mansour, 24, is one of the community health workers in Za'atari who goes tent to tent every month to deliver two drops of oral polio vaccine (OPV) to all children under age 5. He was a nurse in Syria before he and his family fled and landed in Za'atari more than a year ago.

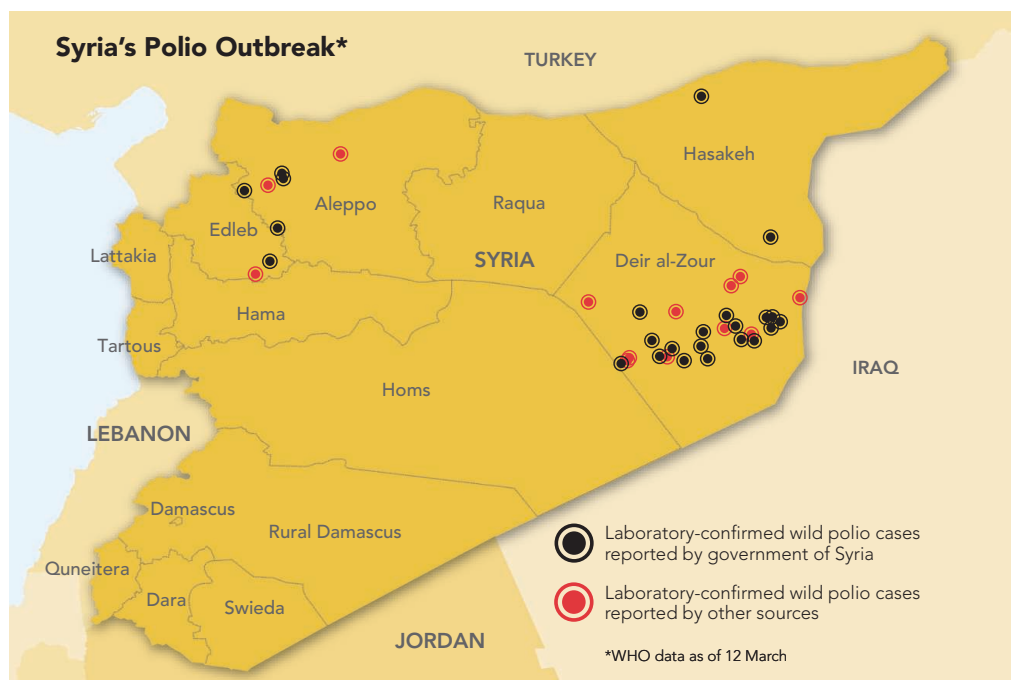
Sitting cross-legged on the carpet, Mansour talks about his life back home and how it, and the country, collapsed. Health care was excellent and affordable and routine immunization rates were high, he says. But with the conflict, “everything stopped.” Hospitals and clinics were destroyed and

health care workers targeted—Mansour says he feared for his life. Vaccines, once plentiful, were no longer available in towns in Dara, a southern governorate where many of the refugees at Za'atari are from. The only way to get vaccine was to make a dangerous trip to a bigger city. “People decided vaccination was not worth risking their lives,” Mansour says.

And now, 15 years after polio was eliminated in Syria, it is back. On 11 October, WHO got word of a cluster of 22 cases of acute flaccid paralysis, or AFP, in Deir al-Zour, a heavily contested governorate in northeastern Syria. Even before conclusive

Pallansch, who heads the division of viral diseases at the U.S. Centers for Disease Control and Prevention (CDC) in Atlanta. But that remains a hypothesis, he emphasizes. (CDC, WHO, the United Nations Children's Fund [UNICEF], Rotary International, and the Bill & Melinda Gates Foundation coordinate the global eradication drive.)

Five days after issuing its initial alert, and before the Syrian government confirmed the outbreak, WHO, in collaboration with UNICEF and the Syrian Ministry of Health, began vaccinating against polio as part of an already-planned immunization campaign



data were in, Aylward, the GPEI director, knew it was polio: The sudden, one-sided paralysis and the geographic clustering “ticked all the boxes,” he told me at the time. WHO issued a global alert on 19 October warning of a suspected polio outbreak in Syria with a very high risk of spread across the Middle East. The first documented case had actually occurred on 14 July in Aleppo, but WHO only learned of it in October, after the cluster was reported. The Syrian government did not announce the outbreak until 29 October.

The virus turned out to be closely related to two known strains, one detected in the sewers in Egypt in late 2012 and the other in Israel in early 2013. No cases have occurred in either country. Both of those strains were quickly traced back to Pakistan. The most recent genetic analyses suggest the virus may have arrived in Syria from Pakistan sometime earlier in 2012, then spread across the country and spilled across its borders, says Mark

against measles, mumps, and rubella. “It wasn’t a great campaign but it probably wasn’t a terrible campaign either,” Maher says. At about the same time, Jordan and other countries strengthened their border vaccination, and the first polio campaign was conducted in Za'atari on 28 October.

A beautiful plan

On 26 October, Mike Ryan was in Dublin, where he teaches international public health at University College Dublin, when he got a phone call from an old friend from WHO. It looked like there was a polio outbreak in Syria, Bruce Aylward told him. WHO needed him urgently in Amman to draw up an emergency response plan for the entire region.

“I couldn’t say ‘No,’” Ryan says. “Bruce and I grew up together as kids at WHO in the ’90s,” says Ryan, a gregarious, red-haired Irishman who led WHO’s global outbreak alert and response network for more than a

decade before leaving WHO in 2011. “Two days later I was on a plane to Muscat,” in Oman, where on 29 October WHO’s Alwan had convened the yearly meeting of all the ministers of health for the Eastern Mediterranean region. The Syrian minister was there, and polio was at the top of the agenda.

Aylward led the polio discussion. A slim, intense man with a shock of sandy hair, Aylward, 50, speaks fast and emphatically. With routine immunization falling below 50%, down from 95% or so before the war, Syria is a polio tinderbox, and several of the surrounding countries—in particular Lebanon and Iraq—do not have strong firewalls. We are dealing with a reinfection of the Middle East, Aylward told the ministers, and whatever their differences, they would have to work together to stop the outbreak.

To reach all children under age 5 in Syria

5 weeks,” says Ryan, where he set up WHO’s one-man polio shop.

The first job was to get the warring factions to cooperate. “The concept that really rang true with all sides of the conflict was that these were children under age 2 who were being paralyzed, who have no part in any conflict. ... Without taking sides we were able to articulate the view that there is one thing that could be done, and this could be done quickly and this could be done effectively.”

By 26 November, Ryan, working closely with UNICEF, various nongovernmental organizations (NGOs), and ministries of health from different countries, had a draft emergency response plan for Syria, Iraq, Jordan, Lebanon, Turkey, the West Bank and Gaza Strip, and Egypt, giving top priority to Syria. Estimated to cost \$40 million in its first phase, the plan clearly lays out what all of

which is aligned with the opposition, was a particular flash point.)

Second, the Syrian government would retain sole authority of the outbreak response. And that meant no collaboration with the opposition or with humanitarian groups that support it, such as the Assistance Coordination Unit (ACU) or the Syrian American Medical Society (SAMS).

U.N. agencies, which operate in Syria by invitation of the government, are bound by those rules. “We are not some independent agency that can waltz in and do what we like,” Maher says. But there’s a problem, he adds. “Cross line has not been working in many of the contested areas.”

One challenge has been getting vaccine into the besieged areas, whether they are besieged by the government or the opposition. Another is ensuring that vaccine reaches all the areas of the north where the radical Islamic State of Iraq and the Levant, known as ISIS, and more moderate opposition factions are fighting each other. Even in areas where the conflict is less intense, the lines of political control are constantly shifting, so it is hard to know who is in charge and which NGOs might be able to get in, Maher says. “If there is a bloke with a gun in your face, you don’t go.”

Into the north

It doesn’t look like a \$40 million operation. At WHO’s polio command center in Amman in mid-January, the phones aren’t working. Maps of Syria are taped to the walls, and the skeleton crew, several of whom have just arrived, are sharing desks. Chairs are in short supply, and the atmosphere is one of controlled chaos.

At 9 a.m., Maher is huddled over a laptop with a colleague who flew in the night before with fresh data on the January immunization round in Syria. Maher wants just one uninterrupted hour with him to figure out how many kids were vaccinated, how many were missed, where, and what needs fixing for the February round. The interruptions are constant. “Sorry about that, mate,” he says after each, grumbling, “I desperately need a technical person.” Maher and his colleague finally reconnect later that night and finish reviewing the data and planning the next round over a bottle of whiskey in Maher’s room.

The next evening in the lobby bar in the Amman Marriott, the team’s home away from home, Maher is fairly upbeat. The January rounds went better than expected, and for the first time vaccinators gained wide access across the opposition-held north. Turkey broke the logjam, supplying vaccine to the north in defiance of the Syrian regime,



Long walk. In January, vaccinators got into the rebel-held areas in the north of Syria.

and across the region, each country would need thousands of vaccinators to go door to door. They would need to quickly license a newer vaccine, bivalent OPV, which works against the two remaining strains of polio and is more effective than the trivalent version the countries relied on when they first wiped out the virus. And not only would the Syrian government have to commit to vaccinating every child in the country—and that meant children in opposition-held areas as well—it would have to do it six times over the next 6 months.

There were a lot of deep breaths and some equivocation, Aylward says, “but no outright ‘No’s.’” On 30 October, the ministers adopted a resolution declaring polio a regional public health emergency.

“The next day I flew back to Ireland and grabbed another bag and went to Amman for

the countries would have to do over the next 6 months to a year.

But reality proved a lot messier. “It is one thing to have a beautiful plan on paper, but it is different when you are fighting a war,” says Ryan, who returned to Dublin on 3 December. “It is not pretty.”

Political minefield

Blunt, with short gray hair and hooded blue eyes, Chris Maher has a reputation for getting things done. When he arrived in Amman in November to relieve Ryan, he walked into a political minefield.

The Syrian regime had laid down two red lines. First, the government would allow vaccine to move “cross line” or into rebel-held or contested areas. But no vaccine could come “cross border” from another country. (Turkey,



On a tightrope. Chris Maher (top), Bruce Aylward, and Mike Ryan (shown in 2003) are treading carefully as they fight polio in Syria and the Middle East.

the humanitarian groups ACU and SAMS have just announced. Some 8500 well-trained vaccinators from local communities went door to door, delivering vaccine and marking every child's little finger to show they were vaccinated, says a SAMS staffer from the Turkey office who declines to be identified. An estimated 1.4 million kids in the north who had not been reached before were vaccinated, he says in an e-mail.

"Now we have a mechanism to get to these communities, and the mechanism is working," Maher says. "With the January round we can say with some confidence that every governorate in the country had immunization activity, and while we may not have reached every community, an awful lot of kids got immunized ... maybe 80% or plus kids got immunized in January, which is bloody good."

The critics

But by dancing with the Assad regime, WHO has opened itself up to criticism. Almost from the outset, NGOs complained that WHO wasn't doing enough to reach

the kids in opposition-held areas.

The accusations escalated in November and December, following articles in the German newspaper *Der Spiegel* and by the news agency Reuters. The news stories contended that WHO and the Syrian Ministry of Health excluded the rebel stronghold Deir al-Zour from a polio vaccination campaign in December 2012, thereby contributing to the outbreak that began there 10 months later, and that WHO was slow in testing stool samples and confirming suspected cases from rebel-held areas.

The polio outbreak in Syria was both "predictable and preventable," wrote Adam Coutts and Fouad M. Fouad, of the London School of Hygiene & Tropical Medicine and the American University of Beirut, respectively, in a 1 January opinion piece in *The New York Times*. Asking "why the international community did not prepare better for this eventuality," they conclude: "A disturbing part of the answer is that the United Nations itself has aggravated the situation" by ignoring Syria's larger health crisis.

Annie Sparrow, a pediatrician and assistant professor of global health at the Icahn School of Medicine at Mount Sinai in New York City, went further in a 20 February piece in *The New York Review of Books* titled "Syria's Polio Epidemic: The Suppressed Truth." She says that by going along with the government, WHO is significantly underestimating the size of the outbreak. In an interview, she puts the current number of cases at 100 "at a minimum," compared with WHO's 37, and says that WHO's long-established practice of defining a polio case by laboratory confirmation is "totally inappropriate" in the midst of a war. The long delay in confirming the July case from Aleppo was "absurd," she adds. If WHO had "been on the ball," she says, it would have picked up the outbreak earlier and, in its key role in GPEI, defied the regime and launched an emergency response across the entire country.

In interviews and written responses, Aylward and Alwan bristle at the notion that WHO in any way contributed to the outbreak, noting that WHO had supported the Syrian Ministry of Health in conducting preventive polio vaccination campaigns since 2010. As for omitting Deir al-Zour in the December 2012 round, Aylward snaps: "WHO doesn't make decisions about who gets vaccinated

where. These are national decisions by governments." In December, violence had flared in Deir al-Zour, so the round was postponed, he says. "And Deir was covered a month later in January [2013]. They always drop that point," he adds.

Aylward concedes the organization has been in a bind because the Syrian government has so far refused to recognize any test results that are not confirmed by its own lab in Damascus or affiliated labs in Cairo and Tunis. Samples from opposition-held areas have gone an unapproved route to an accredited lab in Turkey and so are not counted on the country's official "line list." "Do we like it? No," says Aylward, who adds that WHO is in "frank discussions" with the government about the policy. Meanwhile, he says, WHO reports lab-confirmed cases from any source and continues to use all information in planning its response.

Aylward says the child paralyzed in Aleppo in July was to the best of his knowledge the first documented case, and there was nothing nefarious in the delay in confirming it. The country had been polio-free for so long that no one expected the disease. He adds: "We are \$10 billion and 25 years into this [trying to eradicate polio]. Do you think we would really not vaccinate kids? Come on."

Surveillance systems are so shattered that WHO is undoubtedly missing cases, Aylward concedes. But still, he adds, even if WHO is missing half of the cases, it looks like the Syria outbreak may not be as explosive as feared. "Maybe this time we got out ahead of the virus," Aylward says, although he admits the virus could surprise them yet.

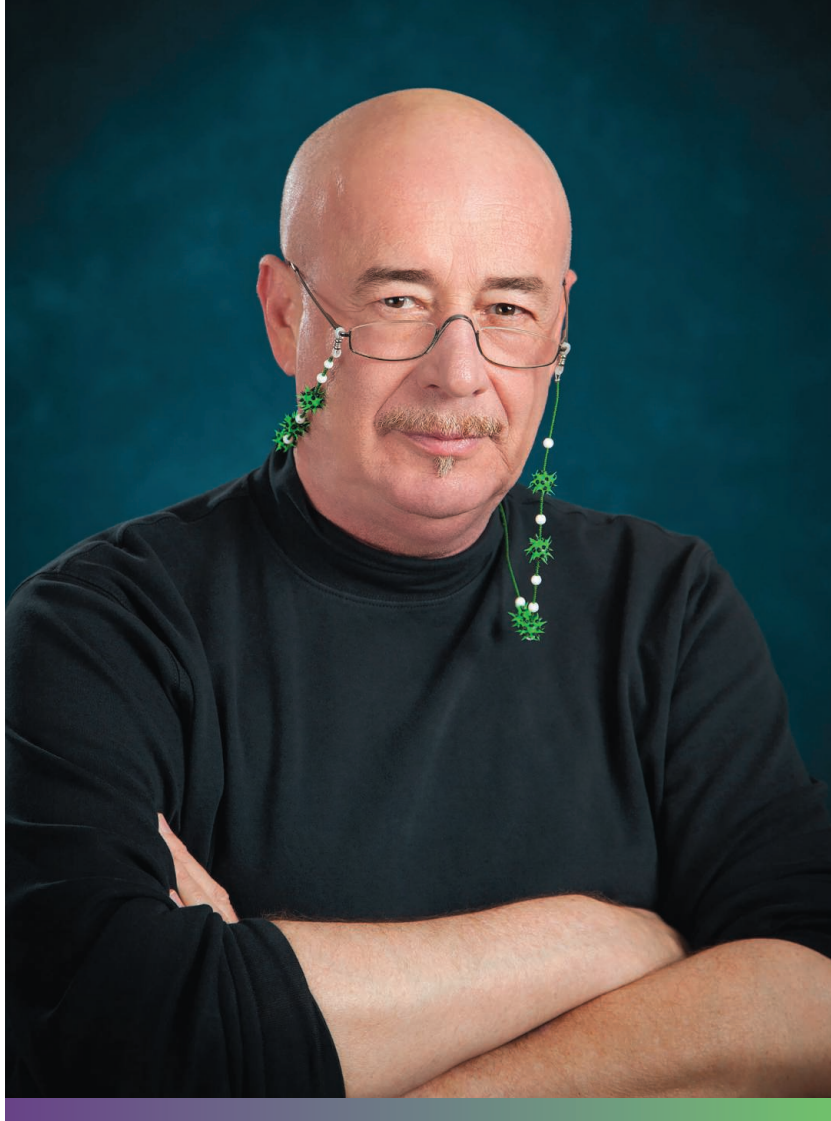
So far four campaigns have been conducted in Syria—three got into the north—and several more are planned. Some 23 million kids across the entire region have been vaccinated multiple times. If the virus doesn't spread out of Syria, the surrounding countries may stop emergency vaccination campaigns after the March to April rounds.

Maher thinks they can quash the epidemic by June, at which point he can go back to worrying about the endemic countries such as Pakistan, which spark these outbreaks in the first place. "Provided we can keep getting into the north and the fighting doesn't screw things up, we've got a shot at controlling this in the next few months."

—LESLIE ROBERTS

"The concept that really rang true ... was that these were children under age 2 who were being paralyzed, who have no part in any conflict."

—MIKE RYAN,
UNIVERSITY COLLEGE DUBLIN



to identify all the functional elements of the human genome, in addition to the 21,000 genes that make up a mere 1% of its 3 billion nucleotides. The co-author of a well-regarded textbook, *Fundamentals of Molecular Evolution*, Graur had been dimly aware of ENCODE's existence until the fall of 2012, when the consortium behind the project announced the first comprehensive results of the 6-year-long endeavor with the simultaneous publication of 33 papers in five journals, including *Nature* and *Science*. ENCODE's signal claim, highlighted by the team in the main *Nature* paper, was that its data "enabled us to assign biochemical functions for 80% of the genome, in particular outside of the well studied protein-coding regions."

ENCODE's leaders drove home that point in videos released by their institutions. "There is not a single place in the genome that doesn't have something that you might think could be controlling something else," said Ewan Birney, the lead analysis coordinator of ENCODE at the European Bioinformatics Institute near Cambridge, U.K., in one of the videos. In another video produced by the National Human Genome Research Institute in Bethesda, Maryland, Michael Pazin, the institute's program director for functional genomics, proclaimed: "Very little of our genomes are junk."

That finding challenged a widely held view, formed after decades of research in evolution and population genetics, that much of the human genome is nonfunctional junk. Other work had already found hints of function in some of the "junk." But Graur found ENCODE's blanket claim patently untrue. To a man of Graur's skeptical constitution, this made ENCODE an irresistible target, a plump duck calling out to a hound dog. Taking the podium in Chicago, he tore into the project.

The heart of his critique was that ENCODE researchers had made an unwarranted leap in the interpretation of their data. The project involved thousands of experiments. In some, researchers exposed cells to a multitude of transcription factors: molecules that bind to genomic DNA to initiate transcription into RNA, the first step in making a vast array of proteins

required for metabolism. In other experiments, researchers identified and inventoried the different RNA molecules produced in various types of human cells. The results showed that more than 70% of DNA in the genome is transcribed into RNA; 8% latched on to transcription factors. Altogether more than 80% of the genome showed some kind of biochemical activity—the basis for ENCODE's claim that 80% of the genome is functional.

That inference, Graur inveighed, was utterly wrong because the mere transcription of a stretch of DNA or the binding to a transcription factor is not a function unto itself. He didn't say it simply; he said it with merciless mocking that, to some, undermined his message. "Graur wrote such a negative paper that it was hard to read," says Bradley Bernstein, an ENCODE researcher at Harvard University. Graur's criticism is so over-the-top that it's not worthy of a response, Bernstein adds. In his Chicago talk, Graur showed a photograph of chewing gum stuck to a shoe as an example of "a function that fits the ENCODE definition." "The fallacy of ENCODE's logic," he said, is this: "We know that some functional regions are transcribed. Ergo, all transcribed regions are functional." Toward the end of the presentation,

The Vigilante

When the ENCODE Project declared that there is no such thing as junk DNA, Dan Graur counterattacked. But does he go too far?

Last year on 11 July, 2 weeks before his 60th birthday, Dan Graur was at the Society for Molecular Biology and Evolution's conference in Chicago, preparing to deliver a scathing criticism of ENCODE, the biggest genomics project funded by the U.S. National Institutes of Health (NIH) since the sequencing of the human genome. An imposing 6 feet 3 inches who likes to wear Hawaiian shirts that flow smoothly over his bulging midriff, Graur speaks with a strong Israeli accent and a deliberate enunciation that lends a scalpel-like sharpness to the sarcasm with which he dissects the world. Besides food and coffee, both of which he consumes immoderately, Graur relishes what he considers to be the unvarnished truth. When a student remarked to Graur—in response to his lament about turning 60—that age was all in the mind, Graur offered a trademark blunt retort. "No," he responded. "It's not in my mind. It's in my knees, my prostate, and my lower back. So go away."

Graur's talk that afternoon was an encore to a paper he had just published with two colleagues assailing the claims made by ENCODE, short for the Encyclopedia of DNA Elements. Launched in 2003 as a successor to the Human Genome Project, ENCODE's goal was

CREDIT: CHRIS WATTS/UNIVERSITY OF HOUSTON

he showed a photograph of dollar bills taped together in the shape of a toilet paper roll—his view of what ENCODE had achieved with the \$288 million spent on the project so far.

Graur isn't the only one who has taken ENCODE to task. Others have made some of the same criticisms, including prominent biochemist W. Ford Doolittle of Dalhousie University in Halifax, Canada, who published a critique in the *Proceedings of the National Academy of Sciences* a month after Graur and his co-authors published theirs in *Genome Biology and Evolution* (GBE).

But if ENCODE has a *bête noire*, it is Graur. "The splashiest part of ENCODE was a conclusion that could not hold up, and Dan pointed

the drizzle, he made a series of sardonic remarks about himself and the world, much as a standup comic might. He said he'd taken to wearing colorful shirts to work because he had been told that his earlier habit of wearing black intimidated students.

Born in Romania, Graur moved to Israel with his family in 1964, when he was 11. He describes himself as being a "goody two-shoes" growing up—a dubious claim in light of the fact that he was thrown out of school in 10th grade for writing off-color jokes in the school newspaper. (His only regret is that the jokes weren't funny.) He went to a technical school to be a lab technician, but was thrown out of there, too, after 2 years for making a political joke. (This one, he claims, was funnier.) He went on to serve in the Israeli army, where one of his field assignments was to lug generators for radio sets during the war with Egypt in 1973.

After his army stint, Graur studied chemistry for his undergraduate degree. Later, after getting a doctorate from the University of Texas, Houston, he taught at Tel Aviv University until 2003, when he turned 50 and grew restless. "At that age, people change their car or wife or computer system," he said. "I changed universities." The move brought him back to Houston, where he has spent the past decade producing papers on genomic evolution, with a focus on the comparative study of genomes. His other passion is collecting modern art, including a number of creations made from household junk. He wears his atheism on his sleeve: One of his pastimes is needling a devout Christian in his department with questions about the veracity of various biblical stories. Another is challenging antiabortion campaigns run by religious groups on campus.

Graur is given to intemperate griping over whatever he finds silly or stupid or wrong.

By his own admission, he has a streak of vigilantism: On occasion he'll produce a serious paper that debunks someone else's finding. In 2001, he and a colleague at Tel Aviv University published a genetic analysis showing that a bacterium claimed to be 250 million years old was likely just a modern strain. Another team confirmed that Graur was right. When we met in December, he was getting ready to publish a study designed to poke statistical and analytical holes in a claim that the last common male ancestor of humans walked on Earth 338,000 years ago. On his personal blog, labeled Judge Starling (Judge is "Dan" in Hebrew; Graur is "starling" in Romanian), he regularly excoriates science in his field that he deems shoddy or hyped.

Graur's atheism inflamed his anger at ENCODE. He perceives an echo of intelligent design in the consortium's "80% claim," which he takes to imply that most of the genome exists because it serves a purpose. "What ENCODE researchers did not take into account," he contends, "is that everything is shaped by evolution." And evolution is slow to weed out useless features.

Genetic mutations—the drivers of evolution—occur at random, and those that are deleterious are weeded out, sometimes over many generations. Other mutations, salubrious and inconsequential alike, get passed down to progeny. As a result, species like humans and elephants that have a small effective population size are expected to accumulate a lot of junk in their genomes.



Line of fire. Claims about ENCODE's findings made by Ewan Birney (holding mic) and other project leaders at a September 2012 press briefing stoked Dan Graur's ire.

it out in a way that was impossible to ignore," says Harmit Malik, an evolutionary geneticist at the Fred Hutchinson Cancer Research Center in Seattle, Washington. "No matter what anybody might think of his style, the points he has raised are very meaningful."

Doolittle, who calls Graur one of the "bad boys of molecular evolution," agrees. "As a reviewer of his manuscript, I did suggest he tone it down a little bit at certain places and he did," Doolittle says. "I think people like Dan are very useful. We simply do not do enough debunking in science these days. We have moved into a very positivist mode where everybody is expected to simply get with the program."

The few ENCODE scientists who've responded to Graur's criticisms say these are off the mark or blown out of proportion. And judging by the continuing flow of funds to the project—\$30 million and counting since September 2012, for characterizing the behavior of genomic elements in more types of human cells—Graur's furious attacks have left ENCODE unscathed.

Faultfinder

I met up with Graur on a rainy day last December at the University of Houston in Texas, where he has been a professor of molecular evolutionary bioinformatics since 2003. When he saw me watching squirrels, which routinely surprise visitors on campus by coming within stomping distance of people to beg for food, he noted dryly that the animals are simply "rats with good PR." Walking through

Various lines of evidence support the idea that vast genomic tracts in many species are littered with junk, he says. One is the surprising lack of correlation between an organism's complexity and the size of its genome. (The onion's genome is five times larger than ours.) Researchers have also discovered that more than 70% of the human genome is interspersed with repetitive stretches of DNA known as transposable elements, which are mostly inactive. Similarly, researchers have identified nearly as many defunct genes and pseudogenes in the human genome as genes.

The true benchmark of functionality, Graur and many others say, is whether a DNA sequence has been conserved over time. Because mutations in functional regions of the genome are likely to impair function, and thereby threaten survival, such mutations are expunged from the population. From this, researchers infer that functional regions evolve much more slowly than the rest of the genome and are conserved; that is, such regions can be expected to show up as identical or similar in genomes across and within species. By sequencing and comparing genomes of different species, researchers have estimated that only 5% to 15% of the human genome is functionally relevant.

To ENCODE researchers like Bernstein, conservation is too narrow a criterion for pronouncing a region of the genome to be functional. But Graur says that view is tantamount to saying that "evolutionary laws governing all known functions in the genome do not apply to the 'functions' defined by ENCODE."

He alleges that ENCODE leaders made such broad claims because they wanted to create a media splash that would justify the project's cost. "They needed to have something big to say," Graur says. "Why did they want to publish all the 30-some articles on the same day? Because they wanted a public relations impact."

Graur contends that ENCODE is an example of how big science can go wrong. "When the average grant size in the biomedical sciences has been halved compared to 10 years ago, this is a scandal," he says. "If you pour \$288 million into one project, you do not fund 500 other projects. You kill the careers of young scientists. They are reduced to becoming technicians."

No meeting of minds

Graur's strong words have struck a chord with some. On his webpage at the University of Houston's site, he has posted some 50 e-mails of endorsement he got from researchers soon after the publication of the March 2013 critique. "Thank you for publishing your paper about ENCODE in GBE," reads one. "[Y]ou proved what many of us thought, but didn't have the time or the courage to state." Since the Chicago conference, Graur says he has received several invitations to deliver his talk on ENCODE. "I seem to have tapped a very big anger," he says.

At the same time, Graur's combative approach has earned disapproval from some quarters. "Would a dispassionate and polite reply have been less visible?" *Nature Methods* asked in an editorial

last fall that slammed Graur for engaging in what the journal saw as uncivil discourse. "Is provocation necessary to get attention from a 'big science' consortium such as ENCODE? We do not think so."

Birney and other ENCODE leaders have not engaged Graur directly. Birney did not respond to multiple requests from *Science* seeking comment on Graur's criticisms. On his blog, however, Birney appears to have backtracked from the use of the term "biological function" in summarizing ENCODE's results. He wrote that ENCODE had revealed 80% of the genome as having "specific biological activity," following up in a subsequent blog post that "we could have used different terminology to convey the concepts, consequence and massive extent of genomic events we observed." The consortium chose the 80% figure—he wrote—because it "brings home the impact of this work to a much wider audience."

Bernstein says ENCODE's value is evident in the hundreds of papers based on project data. As an example, he points to a paper in the *American Journal of Hematology* last November reporting the discovery of mutations associated with X-linked sideroblastic anemia. The mutations—identified through the genetic study of five families that suffer from the congenital disease—are located within a stretch of "junk" DNA that ENCODE had highlighted, which is now known to enhance expression of the *ALAS2* gene.

Graur dismisses that example. The mutations were not discovered because of ENCODE, he points out. After their discovery, the researchers found that the mutations' location was on ENCODE's long list of sequences with some biochemical function. "So what?" he asks. "ENCODE claimed that 80% of the genome is functional. Therefore 80% of all truly functional elements that have been discovered or will be discovered will be found in ENCODE by chance alone."

Graur and other critics place undue emphasis on the 80% figure, says John Stamatoyannopoulos, an ENCODE principal investigator at the University of Washington, Seattle.

The real take-home lesson, he says, is that "there is a tremendous amount of activity encoded in the genome"—much more than researchers had suspected.

Given the current state of knowledge, Stamatoyannopoulos says, scientists need to remain "fairly agnostic" about the potential function of various genomic elements. In other words, while the likes of Graur are asking, How do you know it's functional? Stamatoyannopoulos and others are asking the opposite: How do you know it's not?

That logic infuriates Graur. "If you don't know a function, assume as a null hypothesis that it doesn't have function, and if you find a function, you'll refute the null hypothesis," he says.

I asked Graur if his detractors were right in calling him rude. He didn't think so; moreover, he felt rudeness was irrelevant to the discourse. "Science is not about abiding by a code of behavior put forward by Miss Manners," he told me. "In science, a strong voice is sometimes needed to fight self-promotion and self-delusion."

—YUDHIJIT BHATTACHARJEE

"Is provocation
necessary to get
attention from a 'big
science' consortium
such as ENCODE?"

—NATURE METHODS
EDITORIAL

"Science is not
about abiding by
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—DAN GRAUR



LETTERS

edited by Jennifer Sills

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We recently found that between 2001 and 2011, in half of all hunted populations, human-caused death of grizzlies exceeded mortality rates deemed sustainable by government biologists. In addition, failure to properly account for uncertainty in estimates of population sizes, poaching rates, and population growth parameters meant that hunting targets might have been too high (2). Surprisingly, despite the ensuing media attention, the government reopened hunting in previously overhunted populations, stating, "[b]ecause we recognize inherent uncertainty in our population and harvest rate estimates, conservative mortality targets are used" (3). Although the government's justification borrowed our recent study's language about uncertainty, their decision ran counter to its conclusions. Moreover, the government came under fire during debate in the provincial legislature (4, 5) for claiming in a press release that another recent study (6) confirmed management sustainability, when in fact the paper made no such claims.

Such outcomes reflect a wider problem that often arises when scientific evidence exposes flaws in preferred government policies. Governments can make "science-based" claims without being held to the same standard of transparency and scrutiny expected from scientific researchers. Similar shortcomings were recognized in the proposed delisting of gray wolves from the U.S. Endangered Species Act (Morell's *News & Analysis*) and badger culling in Great Britain for disease control (1). Given the substantial economic and ecological costs of management failure, it is alarming that purported scientific management often proceeds without the hallmarks of science—transparency, intelligibility, and rigorous evidence.

We propose that wildlife managers be held to the same level of scrutiny as research scientists through independent oversight similar to the peer-review process. This would incorporate science into management, ensure that the best available evidence is used in management decisions, and improve accountability to the public for whom wildlife are ostensibly managed.

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Graduates who pursue postdoctoral positions in academia are often on exchange visitor J-1 visas that grant, by definition, temporary and nonimmigrant status. In the industry, H-1B visas can lead to employer-sponsored green cards, but the number of available visas is drastically short of demand, as is the number of available employment-based green cards for people born in certain backlogged countries such as India and China.

Moreover, despite the critical role of innovation and job creation in growing our nation's economy, graduates who start companies—whose products may spring from work accomplished at American universities—face the tallest hurdles. Legally, international graduate student entrepreneurs are often not allowed to found their companies under their own names and, instead, must be sponsored to work for a company owned by another party. An alternative pathway for entrepreneurs is a foreign investor EB-5 visa, but the personal investment of up to \$1 million required from applicants places this option out of reach for most recent graduates. Meanwhile, other countries have

created programs to capitalize on entrepreneurs who were trained in the United States but cannot find a way to stay (*1*).

From a practical standpoint, these restrictions dissuade future innovators from pursuing careers in this country and thereby encourage the development of new technologies elsewhere in the world. Some straightforward reforms that would improve the situation include: (i) authorizing dual intent for student visa holders, (ii) increasing the number of H-1B visas available to recent graduates and increasing the number of green cards awarded to highly educated immigrants in STEM fields, and (iii) creating a new immigration pathway to encourage entrepreneurship.

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Supporting the Scientific Diaspora

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Scientists working abroad, such as myself, often maintain strong personal and professional connections with our home countries. We have linguistic and cultural sensitivities that enable us to serve as bridges and foster trust between cultures and nations (*1*). This can pave the way for long-term relationships.

Letters to the Editor

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In many disciplines, from field biologists to engineers, scientists working abroad

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Research Fellowship aim to provide applicants experience in a novel environment, thus explicitly excluding endeavors in applicants’ countries of origin. Non-U.S. citizens are severely limited in funding options before the principal investigator stage. These deficiencies should be recognized and compensated with complementary programs that encourage interaction with countries of origin. Strengthening the capacity of diaspora scientists to do what we are uniquely positioned to do would go a long way toward meeting global challenges.

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SOCIAL SCIENCES

To Connect More Equitably

Emily Brennan

Women in Minnesota can receive free screening for breast cancer if they do not have any health insurance or are underinsured. In recent years, efforts to make women aware of the free mammograms included a peer-to-peer referral program. “Refer a Friend” encouraged women who had received a mammogram to nominate family and friends who they thought might like to receive some information about the service. Although most women chose not to make any referrals, a close look at the characteristics of those who did revealed some interesting patterns. Referrals were more commonly received from women who had some form of health insurance than from those who were uninsured (1). Referral rates were also higher among those living in neighborhoods with a more stable population and a greater number of religious congregations, two indicators of the strength of social ties within the community (2).

In this case study, we see an example of the type of information-sharing disparities that concern Brian G. Southwell in his timely *Social Networks and Popular Understanding of Science and Health*. As Southwell (RTI International) explains, social networks—that is, groups of individuals who are connected by formal or informal relationships with one another—are one of the fundamental organizing structures of societies. The role of social networks in spreading information has been acknowledged (to some extent) for decades now. Less well understood are the ways that this information diffusion can occur unevenly across networks. In Minnesota, for example, the chances that underinsured, low-income women heard about the free mammography service were influenced by who they knew—or, more specifically, by the socioeconomic status and geographic location of who they knew.

The reviewer is at the Annenberg School for Communication, University of Pennsylvania, 3620 Walnut Street, Philadelphia, PA 19104, USA; after 1 May 2014, at Cancer Council Victoria, 615 St Kilda Road, Melbourne, Victoria 3004, Australia. E-mail: ebrennan@asc.upenn.edu

Southwell brings together much of the social science research that has documented the ways in which information exposure and engagement are shaped by characteristics of individuals (who we are), networks (where we are), and messages (what information). He demonstrates the potential for disparities in what, if any, science- and health-related information people are thinking about and discussing on a daily basis. Well-documented examples of such disparities have shown that they are associated with discrepancies among groups in terms of what is known, what ideas are most salient, and what behaviors are subsequently adopted. Of central concern, however, is the possibil-



ity that these disparities will increase as we move further into the information age.

Here we reach the core of Southwell's argument: increased use of social networks for information diffusion has the potential to exacerbate rather than lessen disparities in science- and health-related outcomes. Despite the widespread enthusiasm for the potential of communication technologies to facilitate information sharing, people (and communities) continue to vary in their opportunities for connecting with one another. At a fundamental level, for exam-

ple, socioeconomic- and geographic-based gaps in access to high-speed Internet persist throughout the United States (3, 4). And, in a more specific example, adults having a history of cancer are less engaged with social networking sites than are those who lack such a history (4).

Yet, not all discrepancies in information exposure and engagement warrant equal concern. Southwell carefully acknowledges the conceptual distinction between differences and disparities. He makes the case that we should be most concerned when disparities stem from unnecessary and unjust structural factors—particularly when these are concentrated among those who historically have been the most disadvantaged.

With this in mind, Southwell identifies three types of initiatives that may, in the long run, facilitate more equitable peer-to-peer sharing. He highlights the need for ongoing investment in both the online and offline infrastructures that enable people to connect with one another. He also proposes efforts to increase collective confidence in both understanding of and willingness to talk about health and science issues—by, for instance, ensuring that people are exposed to information in conjunction with others. The success of public education efforts depends crucially on achieving mass exposure, and Southwell identifies one reason why this is so, collective confidence: Whether people in a community “feel as though they can seek information from others and discuss relevant information with others” likely depends on the extent to which multiple members of the group feel they understand the issue. In a closely linked suggestion, he calls for science and health educators to “meet people where they are” by ensuring that their messages are accessible, usable, and relevant to the everyday lives of their audience.

Southwell has made major contributions to our understanding of the roles that social processes play in driving the diffusion and impact of science- and health-related information. Concise and empirically grounded, *Social Networks and Popular Understanding of Science and Health* cautions against widespread reliance on peer-to-peer sharing strategies to disseminate vital information. In doing so, it challenges all of us—scientists, educators, and policy-makers—to think more critically and creatively about the potential for our communication efforts to increase disparities

Social Networks and Popular Understanding of Science and Health: Sharing Disparities

by Brian G. Southwell

Johns Hopkins University Press, Baltimore, and RTI Press, Research Triangle Park, NC, 2013. 145 pp. Paper, \$24.95, £16. ISBN 9781421413242.

in access to information that can help people live healthier lives in a healthier world.

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EXHIBITION

Wonders from Cambridge Collections

Deborah Dixon

Two Temple Place, built for William Waldorf Astor in 1895 as his estate office, retains many of its original Gothic features. Designed to showcase Astor's art, books, and musical instruments and topped with a gilded weather vane representing Christopher Columbus's *Santa Maria*, the building provides a wonderful backdrop to the Discoveries exhibition. Bringing together objects from eight University of Cambridge museums (1), the exhibition highlights art, science, and exploration as separate themes. It also strives to illustrate how these were historically intertwined and, further, how curation can produce new arrangements of objects that allow visitors to "discover" connections and frames of reference—in particular, ones that cut across the art-science divide.

The collections of the eight museums comprise over 5 million objects. On the exhibition's ground floor, visitors encounter a select few, each chosen for its capacity to inspire curiosity and wonder. For example, Thomas Akilak's *Drum Dancer* (1987), an Inuit sculpture carved from gray serpentine and caribou horn, sits across the room from ten butterflies pinned inside an original wooden display case. These were



Composite dodo.

the models for one of geneticist Reginald Punnett's color plates in a text that sought to suture Darwin's evolutionary theory with Mendel's rules of inheritance (2).

The exhibition extends into the stairwell, where stands a metal reproduction (2003) of Watson and Crick's model of DNA, and up onto the first floor. There, we find a more concerted effort to place objects into groups that reveal both the messy geographies of cross-cultural contact within an imperial context and the diverse fieldwork and representational practices that defy easy categorization as either art or science.

Sample pages from Wyatt Rawson's Arctic diary (1875) and Alfred Haddon's letters to his son from the Torres Strait Islands (1889) provide fascinating glimpses of the field notes and sketches

that together helped explorers make sense of unfamiliar and complex places. A more visually striking group of objects juxtaposes the *Discovery* telescope (which traveled on two polar expeditions aboard ships of that name, as well as on the space shuttle *Discovery*) with scripturally inspired oil color prints (published in 1846) by Isaac Frost of the Muggletonians (a group which held that Earth was in fact stationary). These pastel-hued images,

each representing a celestial logic, are in turn confronted with James Nasmyth's chalk drawing (mid-19th century) of the Moon's crater Copernicus, based on observations from his garden telescope; W. Watson and Sons' sophisticated home telescope (1910), clockwork-driven to compensate for Earth's rotation; and two sublime prints by Sophy Rickett. Her *Observation 95* (Pluto) and *Observation 123* (comet Hale-Bopp), both created in 2012 using photographic negatives taken by astronomer Roderick Willstrop in the 1980s, conjure a sense of infinite space and deep time. Together, these objects mark the ever-expanding horizons revealed by the enhanced gaze of scientists, but they also reveal a desire to animate these remote reaches with colors, shadows,

and textures that render them meaningful.

For many, the exhibition's highlight will be the opportunity to view objects that have never before been on display and will soon return to museum archives. These include a dodo (*Raphus cucullatus*) skeleton, assembled from subfossil bones gathered around 1870 from the Mare aux Songes swamp by Mauritius islanders. Held together by wire, the milky-brown remains perch on a pedestal that raises the dodo's head high above the ground, confronting visitors with a hollow-eyed stare. Next to this weighty and melancholic presence, on its own pedestal, a tinamou (specifically, *Nothura maculosa*) egg collected by Charles Darwin from Maldonado, Uruguay, during the *Beagle*'s voyage, looks very small and fragile. In contrast to the dodo, which became extinct in the late 17th century, tinamous survive today, although several species are vulnerable because of loss of habitat. We are led to wonder, will we soon reach the "last chance to see" moment for them? Through its engaging juxtapositions, Discoveries should spark many reflections.

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Carbon Market Lessons and Global Policy Outlook

Ongoing work on linking markets and mixing policies builds on successes and failures in pricing and trading carbon.

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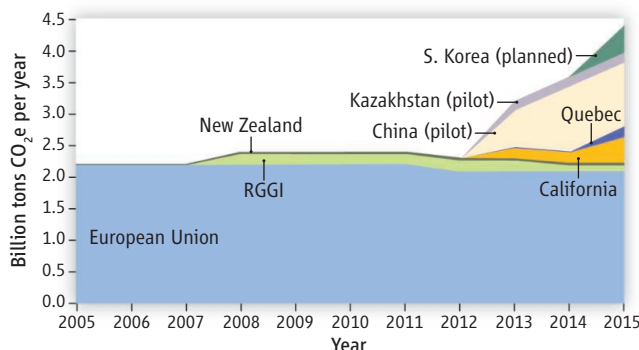
Prices in the European Union's (EU) Emissions Trading System (EU-ETS) spent 2013 at historic lows. Elected officials have promised to repeal the Australian carbon market. Yet five new regional carbon markets recently began in China, which nearly doubled the volume of emissions covered by trading programs. This follows California's successful launch of its cap-and-trade program in 2013 and its 2014 link to Quebec's market. Are carbon markets seriously challenged or succeeding and on the rise?

Sixteen years after the Kyoto Protocol was signed and the idea of emissions trading emerged as a dominant policy paradigm, we have learned much about what makes carbon markets work—and what does not. New questions are emerging for researchers and policy-makers, including how carbon markets fit into a complex global framework.

Think Global, Act Local?

A truly global trading program is as yet unlikely, if not impossible. Because a unit of carbon dioxide (CO₂) emitted anywhere has a uniform impact on global climate, a single global market would be economically desirable, equalizing incentives to reduce emissions everywhere. In practice, we see a multiplicity of multinational, national, and sub-national markets—including the European Union, California, Quebec, the Regional Greenhouse Gas Initiative (RGGI) in the U.S. northeast, and New Zealand, as well as pilot programs in Kazakhstan and China, and programs under development in South Korea and Mexico (see the graph).

Might these programs merge into an integrated global market? This is one of the most important questions facing researchers and policy-makers. Whether, how, and when should markets link together so that regulated entities in one region can use allowances



Estimated annual emissions subject to existing, pilot, and planned carbon markets. CO₂e reflects inclusion of non-CO₂ greenhouse gases converted to an equivalent amount of CO₂ based on radiative forcing. See (2) for details.

or credits from another, thereby equalizing prices (1)? The answers are not easy, as economic arguments in favor of linking must be weighed alongside concerns about environmental integrity, harmonizing politically sensitive program features, and financial flows arising from international carbon trade.

A positive price on carbon in every existing program suggests that markets are reducing emissions below what they would otherwise be. If emissions were not being constrained by the carbon market, then emission allowance supply would outstrip demand in aggregate, and the price would move toward zero. Research on the extent of climate change mitigation, however, remains limited (in part, because complex modeling is required to estimate the “no carbon market” counterfactual). A simple calculation suggests a global, emission-weighted average carbon market price of \$6.50 per ton in 2013 (2). On the basis of previous modeling studies, this price suggests a modest 1.5 to 3.5% decline in covered emissions (3), an annual reduction of about 40 to 90 million tons of CO₂ attributable to existing carbon markets (2).

Greater emission reductions and carbon prices well above these levels are necessary to meet the most commonly identified environmental goals (4, 5). Higher prices are also justified by economic analysis. The most recent U.S. government estimate of the global damages from climate change produced a central value of about \$35 per ton of CO₂ (6). This “social cost of carbon” synthesizes scientists’ best estimates of climate change impacts—

and economist’s valuation of those impacts—from one ton of CO₂. When market prices are below the social cost of carbon, it suggests that higher prices are warranted to fully charge sources for the impact of their emissions.

Part of the story behind low carbon prices is that emissions trading is increasingly only one of several overlapping programs encouraging mitigation. For example, major incentive programs for renewable energy and energy efficiency exist in California and the European Union. These other policies produce targeted emission reductions, leaving the broader carbon market to achieve the (now lesser) remaining reductions. This tends to lower the carbon price but increase overall mitigation costs (4, 7). This raises a question: How do policies work together to achieve goals that extend beyond reducing emissions at the least cost?

Rents, Leakage, Uncertainty

Emission allowances can be auctioned by the government, given to firms (free), or some combination. In the early phases of the EU program, some power producers received carbon allowances at no cost in an attempt to limit consumer price increases. However, depending on the structure of power markets, utilities were able to pass on the market price of the allowances to their customers (8, 9). This was predictable to economists, although not warmly received by the public, who saw companies receiving “windfall profits” from higher electricity prices. These “rents,” net profits received on allowances, are ultimately paid by someone, in this case, consumers. As a result, most carbon markets now limit or eliminate free allocation of allowances to the power sector and instead return revenues from allowance sales to public coffers.

Some programs have attenuated these power price increases, reducing both rents and associated end-user impacts. This can be accomplished in a variety of ways, including renewable energy and efficiency policies that take the burden off the carbon price (10). Lower power prices have the

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downside of reducing conservation incentives, and overall economic efficiency in turn, however.

Many stakeholders have expressed concerns about economic competitiveness, e.g., that energy-intensive industries facing outside competition will relocate to places without a carbon price. This concern has an environmental angle, emissions “leakage”: that emission reductions may simply be shifted outside carbon market boundaries. Evidence seems to indicate that competitiveness impacts and leakage have thus far been small (11). Carbon prices have generally been modest. In many programs, heavy industries have received free allowances to compensate for increased production costs. The extent of competitiveness and leakage impacts, as well as pressure to address them, will depend on the future size and persistence of carbon price differences across political boundaries.

Carbon markets face substantial uncertainty over prices. Although market prices are relatively modest, program designers seek to prevent allowance prices from exceeding economically and politically tolerable levels. Others have been concerned about unexpectedly low prices undermining mitigation investments, technology development, and long-term environmental outcomes.

Allowance banking is an important tool to avoid short-term supply-demand imbalances and associated price movements, such as occurred because of allowance oversupply at the end of 2007 in the EU-ETS. Allowances issued in 2007 could not be banked for use in 2008, and their price fell to zero even as 2008 allowances traded at more than €25. All carbon markets now allow banking.

Price floors have also been used successfully in the RGGI and California programs to avoid lower-than-desired prices. Both programs employ minimum allowance auction prices to withhold allowances if the market is unwilling to pay the minimum auction price.

Both programs also have limited mechanisms to address high prices. Each maintains a fixed allowance reserve that can only be tapped if buyers are willing to pay an established ceiling price. An open question is how large a reserve is necessary, but there is no reason to believe that price ceilings—with a large enough reserve—would be any less effective for avoiding high prices as floors have been for avoiding low ones (12).

Linking, Mixing, Revising Policies

We have alluded to two new challenges confronting domestic policy-makers: linking programs and mixing multiple policies. Each also relates to larger international issues.

Beyond bilateral links, we might ask what can or should be done to facilitate linking multilaterally? The biggest linking story so far was not between two trading programs, but between the EU-ETS and the Clean Development Mechanism (CDM), the United Nations (UN)–sanctioned offset program in developing countries. Over a billion tons of CDM credits have been purchased for compliance in the EU-ETS. Offset projects reduce emissions or absorb carbon and are undertaken by parties not required to obtain permits from the carbon market (13). When linked to a trading system, offset credits can be used for carbon market compliance.

There are a variety of challenges faced by offset programs, and the market for CDM credits has essentially collapsed due to a supply-demand imbalance (11). However, two strengths of the CDM are that it (i) was sanctioned by the UN Framework Convention on Climate Change and (ii) focuses on developing countries where financial flows arguably raise fewer issues. For example, in the California-Quebec link, Quebecois may question if they should collectively send money to California to pay for emission reductions. Such concerns might be more muted if funds were flowing to poorer countries.

This suggests there is value in approaches that simultaneously facilitate carbon markets in developing countries and links to existing programs in developed countries. For example, this might occur through negotiation of “model rules” for domestic trading programs.

The mixing of multiple carbon policies within countries also raises international issues, including how countries can compare one another’s policy portfolios (14). For jurisdictions with carbon markets, comparability is a prerequisite to any potential linkage. The ability to compare emissions reduction efforts is also necessary to justify continued domestic action because of concerns over competitiveness, emission leakage, and the imperative that global reductions ultimately require effort from all major emitters.

Climate policy and carbon markets are constantly evolving. For example, development of shale gas in the United States and subsequent expansion of natural gas generation was one of the drivers behind the RGGI states’ decision to propose a 45% reduction of their cap level (15). Improved representation of abrupt climate change and sea-level rise were among the climate science updates that led to an upward revision of the U.S. government’s social cost of carbon by nearly 40% (6). Revisions to carbon market policies are essential to long-term efficiency (12).

Although markets and stakeholders crave certainty, governments cannot guarantee it.

Although policy revisions cannot be avoided, there is value to governments striving to make them transparent and orderly. Regulatory agencies, courts, legislatures, and central banks need to make market-sensitive decisions while allowing market participants equal access to information. With orderly and predictable policy changes, it is possible for markets to incorporate scientific and technological developments into the carbon price before the policy changes occur (12).

Carbon markets are now a key part of an emerging, complex, global policy framework that mixes trading programs and other policies at the subnational, national, and multinational level. Fresh research and policy initiatives are grappling with new issues: linking programs (16), the consequences and comparability of mixed policies (17, 18), and managing market evolution as policies inevitably change (19). The future of carbon markets will depend, in part, on how well such efforts address these and other challenges.

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Supplementary Materials

www.sciencemag.org/content/343/6177/1316/suppl/DC1

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Testing the Limits of Accretion

Andrew King

Many bright astronomical systems produce their light through accretion. In this process, matter falling toward a gravitating object (a star or a black hole) gives up its gravitational binding energy to electromagnetic radiation. In practice, the matter always has angular momentum, so the infall occurs through a sequence of near-circular orbits of decreasing radius and specific angular momentum, forming an accretion disc. The total radiation output of the disc depends on the rate at which matter spirals inwards and the depth of the effective gravitational potential well around the accreting object. Black holes have the deepest potential wells, and so are the brightest astronomical systems at all mass scales. Supermassive black holes power quasars, and stellar-mass black holes power the most luminous x-ray binary systems. On page 1330 of this issue, Soria *et al.* (1) discuss observations of a bright stellar-mass binary system, called MQ1, in the galaxy M83. Focusing on the fact that there is an expected limit to the total luminosity of any system held together by gravity, they find that MQ1 may exceed this limit, thereby suggesting a rethink on present accretion models.

The more luminous an object, the greater the outward photon pressure opposing gravity. Therefore, a star of a given mass will blow itself apart if its luminosity exceeds a critical value, specified by its mass. This critical luminosity is known as the Eddington limit, and the most luminous stars lie close to it. Although Eddington discovered his limit for the case of a star in hydrostatic equilibrium, it is clear that a similar limit must apply to objects powered by accretion, because eventually radiation pressure must inhibit the infall generating the radiation itself. In general, this seems to be largely true. However, MQ1 appears to be an exception, with a power output exceeding the Eddington limit.

To reach this conclusion requires both a mass for the accreting black hole in MQ1 and an estimate of its power output. Because MQ1 is a microquasar, a straightforward estimate of the power can be made. Microquasars are bright black-hole binary systems that inject large amounts of mechanical energy into the



The W50 nebula surrounding the energetic system SS433. The powerful wind from this object has probably inflated the central spherical part of the nebula, whereas the precessing near-relativistic jets have inflated its "ears."

tenuous interstellar gas surrounding them, most noticeably in the form of narrowly collimated jets, but also, and often more powerfully still, through roughly spherical winds. The prototype of these systems is the binary SS433 in our own galaxy (see the figure). Spectroscopy shows that the two opposed jets have speed 0.27 c (c is the speed of light) and precess on a cone with a period of 162 days, whereas the wind has a velocity of 1500 km per second. Both wind and jets leave their distinctive imprints on the surroundings, the first producing the large (~40 parsecs) near-spherical nebula called W50, whereas the precessing jets inflate the "ears" of the nebula. The theory of how winds can inflate spherical bubbles of this kind is well understood and allows an estimate both of the total energy injected by the wind and how long this has been happening. Dividing the first number by the second gives an estimate of the average mechanical power that the wind has injected. For MQ1, this gives a power of $\sim 10^{40}$ ergs per second (erg/s), similar to what is found for SS433 and a number of other sources.

The unique feature of MQ1 is that its x-ray emission is dominated by blackbody emission from its innermost accretion disc. Comparing the blackbody temperature with the x-ray luminosity gives a characteristic

Observations reveal a greater influence of rapidly accreting black holes on their host galaxy than expected.

area, which translates to a black hole on the order of 10 solar masses. Under normal circumstances, this would predict an Eddington luminosity of only 10^{39} erg/s, implying that at least MQ1's mechanical output was substantially super-Eddington over the 10,000-year history of its nebula. Although the Eddington limit explicitly applies to radiative output, not mechanical, it is difficult to imagine that a highly dissipative system such as an accretion disc could have avoided rough equipartition between mechanical and radiative luminosity. MQ1 appears to have violated the limit, and Soria *et al.*'s observations give the first direct instance of such a conflict.

But there is another possibility. In their classic study of the physics of disc accretion, Shakura and Sunyaev (2) asked what would happen if mass was supplied to the disc so rapidly that it would generate a luminosity far exceeding the Eddington limit. This would mean that, at large distances from the black hole, the gravitational energy release is already super-Eddington.

Shakura and Sunyaev made the simple assumption that the excess accretion (above what would locally produce the Eddington luminosity) would be blown away by radiation pressure at each radius. The resulting disc has two striking properties. First, a

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near-spherical wind carries away most of the original mass, and second, a total luminosity exceeding the Eddington limit by a dimensionless factor. This factor increases as the natural logarithm of the ratio of the actual mass supply rate to the rate formally giving precisely the Eddington luminosity—about 10^{-7} solar masses per year for a 10-solar mass black hole as in MQ1. In moderately super-Eddington cases, this ratio is of order unity, so the logarithmic factor is itself unity. To reach values of order 10 or so, as for MQ1, requires mass supply rates of order 5 to 10 thousand times the critical rate, i.e., several 10^{-4} solar masses per year (3). This is a truly enormous rate, as it would deplete the mass of the companion star in a very short time (10,000 years or so).

Yet just this case does occur in SS433 (4), and may well occur for MQ1. In both cases, we have an independent check, in that most of this huge mass supply ends up as the wind expanding the surrounding nebulae. This does appear consistent with the size and age of the nebula in both cases. The cause of the huge mass supply rate in these two binary systems is that the companion star is prob-

ably more massive than the black hole and expanding for internal evolutionary reasons. When some of its mass falls into the disc around the black hole, this means that it must have gained angular momentum, as it is now farther away from the binary's center of mass. To conserve angular momentum, the binary system has to shrink, despite the fact that the donor star is trying to expand. This causes the mass transfer rate to rise to very high values, consistent with those inferred for SS433 and now MQ1 (4). Stabilization occurs once the mass transfer has reversed the mass ratio, when further mass transfer makes the binary expand to conserve angular momentum, thus reducing the tendency of the donor star's gas to overflow toward the black hole. This may have recently happened in MQ1 but not yet in SS433.

Soria *et al.* provide a clear example of what happens in hyperaccretion, when the mass supply greatly surpasses the rate giving the Eddington limit. This state may be implicated in most ultraluminous x-ray sources (ULXs), because another effect of the huge mass outflow is that much of the radiative luminosity is forced to emerge through

two narrow funnels along the accretion disc axis close to the black hole, and so is closely aligned to the jets. An observer viewing the system along these special lines of sight, but assuming that the radiation was emitted isotropically, would infer a huge luminosity (5). For SS433, we see the jets from the side rather than head-on, so it is clear that we are not viewing it from these special directions, and it does not appear as a ULX. As Soria *et al.* remark, for MQ1 this is less clear, so this system might have appeared as a ULX in the past. It seems likely that this system has much to tell us about the relationship between microquasars and ULXs, as well as how hyperaccretion works in practice.

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CHEMISTRY

Nanoframe Catalysts

Julia R. Greer

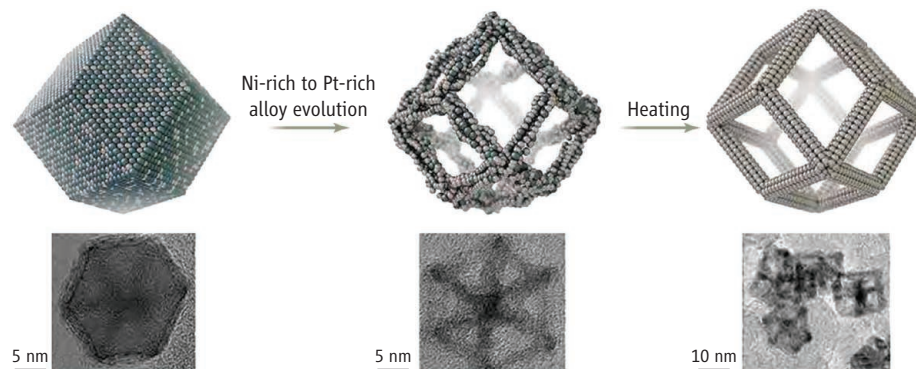
The catalytic properties of materials depend strongly on their microscopic structure, with the atomic-level chemistry and structure directly influencing the activity and durability of the catalyst. However, these microscopic properties can be difficult to understand and control. Furthermore, most efficient catalysts contain substantial amounts of precious metals, rendering them prohibitively expensive. The search for efficient, inexpensive catalysts has, therefore, been challenging. On page 1339 of this issue, Chen *et al.* (1) report the synthesis of a new class of electrocatalysts built from platinum-nickel nanocrystals. Their Pt_3Ni nanoframes have more than 22 times the catalytic activity of conventional platinum/carbon catalysts at 0.9 V, yet contain about 85% less precious metal.

Platinum is one of the best electrocatalysts for various applications that require

oxygen reduction, including metal-air batteries, fuel cells, and alkaline water electrolysis (2, 3). However, the cost of this precious metal prevents its use in the mass production of these devices. Substantial research efforts have been dedicated to developing electro-

Nanoframe electrocatalysts containing only small amounts of precious metal show very high activities.

catalysts that use much less platinum without sacrificing efficiency. One way to achieve this is to alloy platinum with non-noble metals. Another approach is to increase the surface area-to-volume ratio by creating hollow and porous nanoparticles. Chen *et al.* combine



How to make nanoframes. In the synthesis reported by Chen *et al.*, initial single-crystalline Ni_3Pt nanoparticles are etched to form hollow nanoframes of the same geometry. When the nanoframes are heated the composition of the nanoframes changes to Pt_3Ni and a platinum-rich "skin" forms on the surfaces of all edges. The open structure facilitates easy molecular access to all surfaces, leading to a much higher catalytic activity than that of conventional platinum-carbon catalysts.

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these two approaches to transform solid Ni₃Pt polyhedral nanoparticles into open-cell three-dimensional (3D) frames that are thermally annealed to segregate most of the platinum on the outer surfaces of the edges in the nanoframe structure.

To produce the nanoframes, the authors first synthesize Ni₃Pt nanocrystals. The initial shape of the Ni₃Pt is a rhombic dodecahedron with dimensions of ~20 nm and single crystal-line face-centered cubic atomic structure. The authors submerge these crystals in a chemical solution that selectively erodes them to form 3D Pt₃Ni nanoframes shaped like open truss structures, with eight facets in each frame (see the figure). The interior faces within the polyhedral are preferentially etched out before the edges, which initially contained the largest relative amount of Pt. In the resulting structure, the surface of each edge is rich in single-crystalline platinum, which facilitates oxygen reduction and hydrogen evolution reactions. The corrosion process that hollows out the nanoparticles occurs spontaneously in air, in contrast to many other syntheses (4). The elegance of this synthesis route stems from the

relatively simple procedure and the precise thermal control over the resulting material, making this process amenable to scaling up for production.

The specific electrocatalytic activity of Pt₃Ni nanoframes was 16 times as high as that of commercial Pt/C electrocatalysts at 0.95 V; this high activity is one indication for the formation of Pt-rich skin on the surface of Pt₃Ni. The open architecture of the nanoframes provides easy access of the reactants to the internal and external surfaces, leading to a mass activity of 5.7 A mg⁻¹ Pt calculated at 0.9 V, while maintaining remarkable durability over 10,000 cycles between 0.6 V and 1.0 V. This mass activity is more than an order of magnitude higher than the U.S. Department of Energy's 2017 target (5).

Materials with open 3D nano- and micro-lattice structures have recently been shown to exhibit improved structural properties, such as higher strength, resistance to failure, damage tolerance, and recoverability (6, 7). These unique effects arise from the interplay between the structural response to deformation and the size effects in the mechanical

properties of materials at the nanoscale (8). The open structure of the Pt₃Ni nanoframes described by Chen *et al.* provides a promising pathway to combine material architecture and nanoscale design. These two concepts enable the production of 3D cellular nanoarchitectures with high surface-to-volume ratios, which lead to high catalytic activity, minimal use of precious metals, and mechanical stability. Future experiments should include mechanical testing, geometry optimization, and exploring effects of geometry on catalytic activity.

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BIOCHEMISTRY

Unlocking Ancient Protein Palimpsests

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Over the past 30 years, ancient DNA studies have grown from a scientific curiosity into a powerful research tool with applications in molecular evolution, archaeology, paleontology, conservation genetics, and forensics. The analysis of ancient proteins has lagged behind that of ancient DNA, but recent developments in high-throughput, high-resolution mass spectrometry (MS) have the potential to provide the accuracy and robustness required for confident and reliable sequencing of ancient proteins. This methodology may allow biomolecular recovery to be extended further back in time, because DNA chains fall apart 10 times faster (1) than proteins (2). Furthermore, mass spectrometry enables investiga-

tion of protein expression specific for different tissues, developmental phases, or biological processes, including disease states.

Although the study of ancient proteins can trace its roots back to 1954 (see the figure), analytical methods were unable to obtain sequence information. Bulk amino acid analysis and antibody-based immunoassays provided only tentative or indirect protein identification of previously defined targets. Edman degradation sequencing (by scission) requires high concentrations of a purified and undamaged target protein. This is often difficult in fresh samples, and is wholly unsuited for the analysis of complex mixtures of degraded ancient proteins.

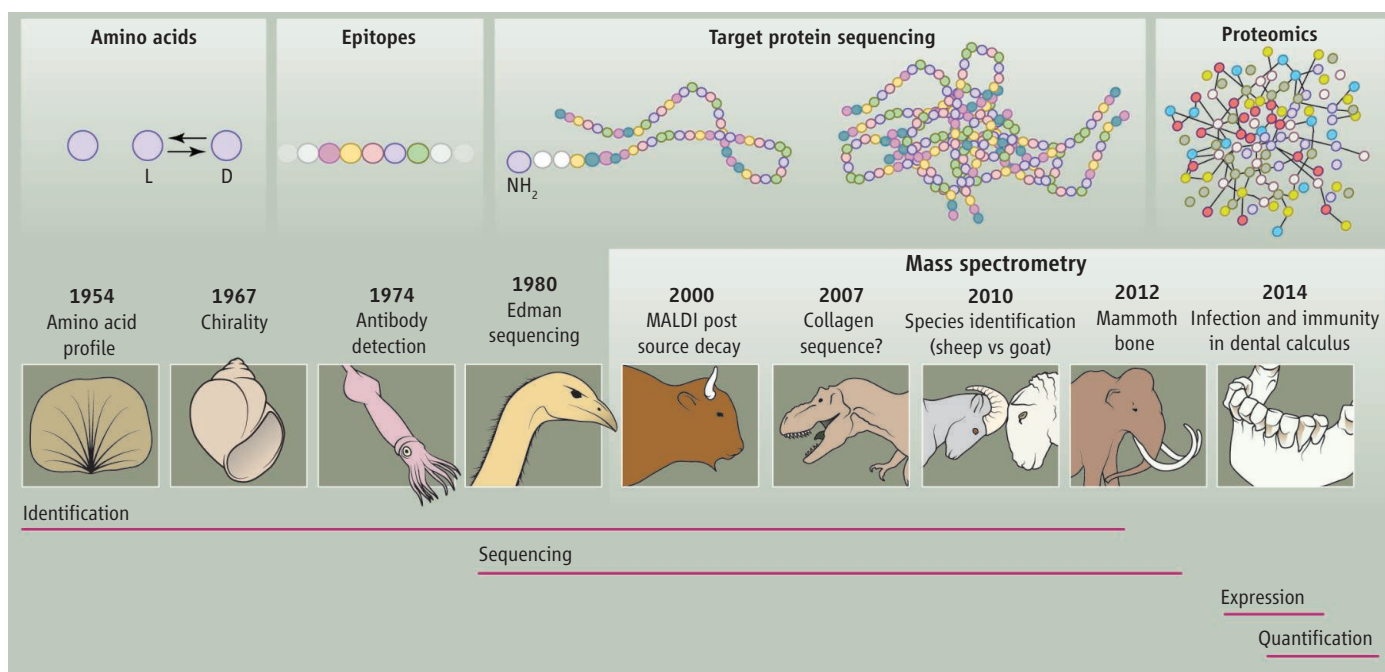
First applied to ancient proteins in 2000 (3), mass spectrometry is particularly well suited to studying ancient proteins, because it characterizes (and quantifies) proteins by identifying multiple short peptides released from complex mixtures after enzymatic digestion. Initial studies focused on the most abundant proteins (osteocalcin and collagen)

High-resolution, high-throughput mass spectrometry studies of ancient protein samples provide insights into protein function in the distant past.

from the most prevalent archaeological fossil material: bone. For example, claims of collagen sequences recovered from dinosaur bones were used to provide molecular evidence to support their phylogenetic placement (4). This approach is now mainly used to identify bone fragments on the basis of collagen peptide fingerprints (5). More recently, attempts have been made to identify multiple proteins in complex mixtures, based on methodological improvements that enable the recovery of up to hundreds of different proteins from ancient samples (6).

For example, shotgun proteomics applied to human medieval dental calculus identified several bacterial highly antigenic virulence proteins, such as gingipains, known to provoke strong immunological reaction. Inflammatory and anti-inflammatory host proteins involved in the innate immune system response, some of which have antimicrobial and bactericidal properties, were also identified (7). In a 500-year-old Inca mummy, a similar approach detected the

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presence of several proteins consistent with severe inflammation and neutrophil infiltration of the airways at the time of death, most probably as a host immune system response to severe bacterial infection (8).

The key technological basis for these advances has been the development and availability of high-resolution mass spectrometers. The latest generation of these instruments can reliably and (relatively) rapidly detect proteins in complex mixtures, even at the limited quantities typical of ancient samples. Despite these developments, ancient protein research is still in its infancy, comparable to ancient DNA research at the turn of the millennium. Methods appropriate for typical modern samples must be modified to take into account the lower concentrations and higher levels of damage found in ancient samples. Such methodological tailoring is now being undertaken; for example, sample preparation has been substantially simplified to minimize losses (9).

As in the case of ancient DNA, it is important to analyze the target and identify or exclude secondary contaminants. DNA and protein contamination, though, are conceptually different. Unlike DNA, proteins are not amplified, and damage in the extracted sample (10) is the first evidence of authenticity. Furthermore, the tissue-specific nature of protein expression provides a powerful additional test of authenticity. As in any mass spectrometry experiment, extensive fragmentation and accurate measurements of precursor and fragment ions are critical for identification. Candidate lists of identified peptides

or proteins should be validated on the basis of strict filtering statistics and manual inspection of the key evidence. Both the raw data and details of search criteria should be publicly available. Sensitive samples should be prepared in spaces that meet standards equivalent to those used for ancient DNA extraction. Last but not least, contamination from proteins routinely used as MS standards can occur in proteomics facilities; evidence for wool (sheep keratins), milk (bovine casein), and albumins should therefore be validated extremely carefully.

Peptide sequences are routinely identified by matching spectra against a protein reference database, usually derived from annotation of the genome sequence of the biological species under investigation. However, ancient genomic data are rarely of sufficient quality to enable confident gene prediction. Instead, MS data sets from extinct species need to be searched against a protein reference database of the nearest available extant relatives; for example, mammoth MS spectra must be searched against the elephant reference protein list (6). This approach will bias against novel sequences, which are the most interesting for pathophysiological and phylogenetic reconstructions. De novo or hybrid sequencing solutions, initially developed for antibody sequencing or to improve peptide identification in unsequenced living organisms, allow for identification of amino acid substitutions not previously reported (11). This approach, although still computationally challenging at the moment, has the potential to provide groundbreaking results.

A short history of ancient protein analysis.

Collagen-like amino acid profiles were first detected in ancient fossil bone in 1954. The detection of immunologically cross-reactive material in 1974 led to a number of antibody-based investigations of ancient proteins. Efforts to directly sequence proteins began with Edman degradation in 1980, but the methodology was not well suited to ancient proteins. In 2000, following the advent of soft-ionization mass spectrometry, the pace of research on ancient proteins increased (3), with an initial focus on the identification and sequencing of single target proteins. Advances in high-resolution instrumentation now allow researchers to explore ancient proteomes (6).

We remain remarkably ignorant of the details of protein degradation. Adoption of tools to search the entire spectrum of known spontaneous chemical modifications affecting amino acids is beginning to uncover more details of decomposition pathways. This in turn will feed information back into studies of long-lived proteins and the processing and storage of proteinaceous materials. Massively parallel DNA sequencing-by-synthesis has revealed patterns of purine deamination, used to support authenticity of sequences. Similarly, researchers are now exploring patterns of glutamine deamidation (10) as a marker of protein age.

The full potential of ancient proteomics will only be realized once quantification of ancient protein expression is achieved. Quantitative proteomics is a growing trend within biomedical research, underpinning the ability to understand cellular function and regulation. The foreseeable application to ancient samples of quantitative proteomics methods

could offer the opportunity to reconstruct pathophysiological phenotypes characterized by specific protein expression patterns and not necessarily hard-coded at the DNA level.

Ongoing developments in the analysis of ancient biomolecules and integration of high-throughput methods to sequence multiple categories of ancient biomolecules have the potential to provide insights into biological processes in the distant past. Ancient DNA analysis remains at the forefront of ancient biomolecular studies (12, 13). Ancient pro-

teomics has the potential to complement this exciting work—for example, to shed light on disease processes that cannot be captured by DNA studies.

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CELL BIOLOGY

RIPK3 Takes Another Deadly Turn

Jianke Zhang¹ and Francis Ka-Ming Chan²

Programmed necrosis, or necroptosis, is a form of cell death with important roles in many inflammatory conditions, such as innate antiviral responses and atherosclerosis. It is a messy process, characterized by cell swelling and eventual rupture that releases contents into the surroundings, triggering inflammation. Initially considered an unregulated response to nonspecific stress, necroptosis is now understood to be controlled by signaling pathways that intersect with the regulation of apoptosis, a well-characterized programmed cell death mechanism. By contrast, apoptosis is relatively neat and does not trigger an inflammatory response. Determining how a cell succumbs to one form of cell death and not the other may guide the development of therapeutic strategies for acute and chronic inflammatory diseases. On page 1357 of this issue, Newton *et al.* (1) elucidate how the two cell death mechanisms intersect, and discover that disrupting one process leads to a surprising consequence—death by the other.

During apoptosis, the cell dismantles itself through internal proteases (caspases) that are activated in response to “death receptors,” members of the tumor necrosis factor (TNF) receptor family. Necroptosis relies on receptor interacting protein kinase 1 (RIPK1) and RIPK3. These enzymes also respond to the TNF receptor, but as well to toll-like receptor 3 (TLR3), TLR4, and the T cell antigen receptor. Cells are sensitized to necroptosis when certain proteins of the apoptosis

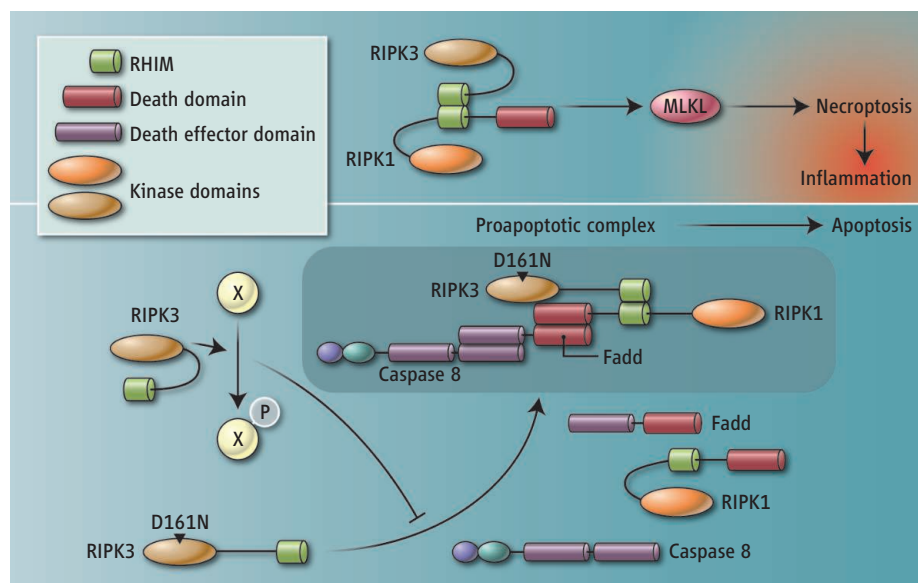
mechanism—caspase 8 or its activator, Fas-associated protein with death domain (Fadd)—are inhibited. In the absence of active caspase 8, RIPK1 phosphorylates and activates RIPK3, which in turn phosphorylates the protein mixed lineage kinase domain–like (MLKL), leading to plasma membrane rupture and necrosis (2).

Studies in mice showed that genetic inactivation of RIPK3 corrected many inflammatory conditions caused by tissue-specific deficiency of Fadd or caspase 8 (2). Similarly, acute injury-induced inflammation, such as that triggered by drugs or ischemia-

Blocking an enzyme involved in necroptosis may prove toxic by promoting another form of cell death.

reperfusion, was ameliorated in mice lacking RIPK3. These findings pointed to inhibition of the kinase function of RIPK3 as a promising therapeutic strategy for acute and chronic inflammatory diseases.

To test the feasibility of this therapeutic approach, Newton *et al.* generated mice genetically engineered to express an enzymatically inactive version of RIPK3 in which aspartic acid at position 161 is replaced with asparagine (RIPK3-D161N). Surprisingly, whereas mice lacking RIPK3 were viable, animals expressing RIPK3-D161N died at mid-gestation from abnormal vascular devel-



Death by association. (Top) RIPK3 associates with RIPK1 to cause cell death by necroptosis and inflammation. **(Bottom)** RIPK3 prevents cell death by apoptosis, possibly by phosphorylating (P) a substrate (yet unknown protein “X”) that blocks the assembly of a proapoptotic complex (Fadd, caspase 8, RIPK1, and RIPK3). However, an inactive form of RIPK3 (D161N) stimulates apoptosis. This may occur through a conformational change that allows the inactive form to promote assembly of the proapoptotic complex.

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opment of the yolk sac. This phenotype was reminiscent of mice that lack Fadd or caspase 8. While mice lacking Fadd or caspase 8 succumbed to extensive necrosis during embryogenesis, the lethality in RIPK3-D161N mice was caused by extensive caspase-dependent apoptosis. Mice expressing RIPK3-D161N and also lacking caspase 8 were normal, but developed a lymphoproliferative disease akin to that caused by inactivating mutations in Fas, a member of the TNF receptor family that stimulates apoptosis. Newton *et al.* show that induced expression of RIPK3-D161N in adult mice triggered the formation of a complex containing Fadd, caspase 8, RIPK1, and RIPK3 that caused massive apoptosis in multiple tissues and lethality. Although RIPK1 is present in this death-inducing complex, blocking its kinase activity did not prevent apoptosis. Moreover, embryonic lethality of RIPK3-D161N mice was rescued by RIPK1 deficiency. Thus, although there is an overlapping set of proteins (Fadd, caspase 8, RIPK1, and RIPK3) that are involved in necroptosis and RIPK3-D161N-induced apoptosis, the molecular mechanisms that drive these two cell death responses are distinct.

How can we reconcile the lethal phenotype of RIPK3-D161N mice when animals lacking RIPK3 were born alive with no overt abnormalities? One explanation is that RIPK3 phosphorylates and inactivates a substrate that prevents assembly of the Fadd-caspase 8-RIPK1-RIPK3 death-inducing complex (see the figure). In this regard, it is note-

worthy that the activities of Fadd, RIPK1, and RIPK3 are controlled by phosphorylation (3, 4). Perhaps RIPK3 directly phosphorylates one or more of these proteins to control the assembly of this RIPK3-associated apoptosis-inducing complex.

Although inhibition of RIPK3 kinase activity is a plausible cause of apoptosis in RIPK3-D161N cells and mice, other mechanisms are also possible. For example, mice that express a single allele of D161N were viable. Because gene dosage is important, the phenotypes cannot be entirely attributed to lack of kinase activity. Previous studies show that a form of RIPK3 in which the kinase domain has been deleted causes spontaneous formation of RIP homotypic interaction motif (5) (RHIM)-dependent amyloid fibrils (6). This indicates that the kinase domain may functionally “mask” the RHIM to prevent inadvertent activation. In this scenario, the D161N mutation could alter the conformation of RIPK3 such that the RHIM is exposed for binding to RIPK1. This model predicts that the kinase and RHIM domains collaborate to control scaffolding of the necroptotic and apoptotic machineries. This type of scaffolding function for kinase domains has been observed with oncogenic kinases such as B-Raf (7). Interestingly, a much lower amount of RIPK3-D161N was expressed compared to wild-type RIPK3, which may be attributed to a conformational change that leads to protein instability. The therapeutic efficacy of RIPK3 kinase inhibitors (8) will depend on

whether they similarly promote assembly of this apoptosis scaffold. In contrast to RIPK3 (9), mice expressing kinase inactive RIPK1 were viable (1). As such, pharmacologic inhibition of RIPK1 may prove to be a more viable option in the clinic.

Although compelling evidence indicates that RIPK3 does not participate in death receptor-induced apoptosis, RIPK3 was originally identified as an inducer of apoptosis (10–12). Newton *et al.* show that regardless of the mechanism, RIPK3 can indeed function as an apoptosis regulator. In that sense, RIPK3 biology has come full circle. The challenge is to determine how RIPK3-dependent apoptosis is induced and whether it has any unique functions in physiology.

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VIROLOGY

Epstein-Barr Virus Turns 50

Paul M. Lieberman

This month marks the 50th anniversary of the discovery of Epstein-Barr virus (EBV) as the first human tumor virus. In March 1964, a team led by Anthony Epstein identified herpesvirus-like particles in cultured tumor cells derived from African Burkitt's lymphoma tissue (1). At that time, the idea that a virus caused human cancer was met with some skepticism because the theory that cancer was infectious had been dismissed in the previous century. Stalwart investigators continued to track EBV until the viral culprit was declared a class I carcinogen by the International Agency for Research on

Cancer and the World Health Organization in the late 1990s. Despite the consensus that EBV is a bona fide tumor virus, the mechanisms of cancer causation by EBV remain an area of active investigation and controversy 50 years since its initial discovery.

One of the most confounding findings relating to the association of EBV with rare cancers is that EBV prevalence in the normal population is extraordinarily high, reaching over 90% of the adult population worldwide. Because EBV is a member of the herpesvirus family, it is very adept at establishing a long-term latent infection. Exposure to EBV can be detected by serology, and latent forms of EBV can be readily detected by molecular methods in a small percentage of B lympho-

Fifty years after the discovery of Epstein-Barr virus and its association with cancer, a vaccine or therapy for the virus remains elusive.

cytes from healthy individuals. Furthermore, EBV was identified as a major causative agent of infectious mononucleosis, which seemed incongruent with its role in cancer causality. How could a relatively common virus be the cause of an endemic childhood cancer in Africa?

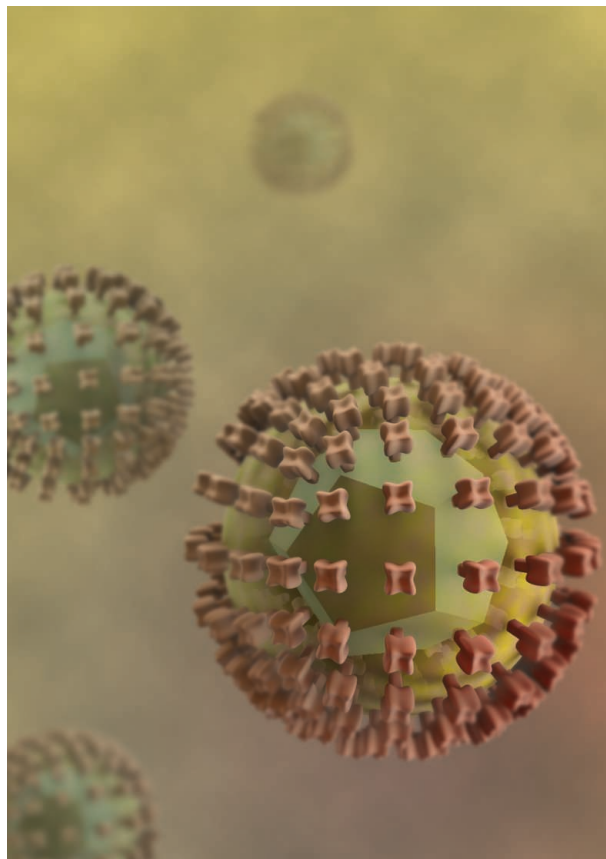
Viral causes of animal cancers had been known since 1911, when Peyton Rous discovered that retroviruses cause cancers in chickens. But it was not until the discovery in 1968 that viruses related to EBV were responsible for T cell lymphomas in nonhuman primates that the case for EBV-dependent tumorigenesis became more compelling. EBV was soon found to be highly efficient at transforming quiescent human B lymphocytes into contin-

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uously proliferating lymphoblastoid cell lines (2), and EBV is now a common laboratory tool used to immortalize B lymphocytes for human genetic studies. The search for additional cancers that contain EBV revealed that latent forms of the virus were present in most nasopharyngeal carcinomas endemic to Southeast Asia. Although EBV is found in ~100% of this cancer type, it was only found in a third of these carcinomas outside of endemic regions, similar to what was found for Burkitt's lymphoma (cancer of B lymphocytes). These imperfect correlations fueled concerns that EBV was not a driver of oncogenesis but merely an opportunistic passenger in cancer, and that high correlations could be attributed to increased viral load in endemic regions.

The discovery of EBV as a causative agent of X-linked lymphoproliferative disease, a rare genetic disorder of immunologic dysfunction, illuminated the importance of host immunologic status in the control of viral-associated malignancies. Moreover, during the era of the HIV-AIDS epidemic and before successful antiviral therapies, the prevalence of B cell lymphomas and Kaposi's sarcoma revealed the opportunistic nature of these malignancies. The massive depletion of CD4 T cells and immune dysfunction in HIV-AIDS is sufficient to unleash the potential of latent EBV to drive immunoblastic large B cell lymphomas. The insight that immunosuppression could drive malignancy fueled the search for a causative agent for Kaposi's sarcoma, and led Yuan Chang and Patrick Moore to identify a second human gammaherpesvirus linked to human cancer (3). Kaposi sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV8), has a near perfect correlation with all forms of Kaposi's sarcoma, and also with some B cell malignancies, including pleural effusion lymphomas. The identification of KSHV as a second human tumor virus from the gammaherpesvirus field solidified the argument that these viruses have a causative role in human cancer.

Infectious agents in cancer were found to be more common than originally thought. In the early 1990s, *Helicobacter pylori* was shown to cause peptic ulcer diseases, a finding that was recognized with the 2005 Nobel Prize to Robin Warren and Barry Marshall. This finding opened the door to linking the



Half-century mark. Epstein-Barr virus was discovered 50 years ago by Anthony Epstein. The details of its association with cancer remain enigmatic, and an effective therapy has yet to be developed.

bacterium with gastric carcinoma. Interestingly, EBV has been consistently found in ~10% of all stomach cancers and is now recognized as a distinct subtype of the cancer. The most compelling case for virus-associated cancer has been made for human papillomaviruses (HPVs) and cervical carcinoma. All forms of cervical carcinoma contain a subtype of HPV that corresponds to a high-risk viral genome. The distinction between low- and high-risk viral genomes provided one explanation for how a common virus could be associated with relatively rare forms of cancer. The high-risk strains of HPV are more likely to develop cancer. The discovery that HPV was the etiological agent of cervical and oral squamous cell carcinomas resulted in a Nobel Prize shared by Harald zur Hausen (2008). To date, the number of viruses or infectious agents associated directly or indirectly with human cancer etiology has grown to include the human hepatocellular carcinoma viruses (hepatitis C and B viruses), T cell leukemia virus (human T-lymphotropic virus I and II), and the Merkel cell carcinoma virus. In all, it is estimated that infectious agents are responsible for one-fifth of all cancers (4).

In addition to being the first human cancer virus to be discovered, EBV was also the first large herpesvirus genome to be completely sequenced (1995), a project that helped launch the genomic era (5). Long thought to have a highly conserved genome with only two major subtypes, recent studies suggest that additional polymorphisms may explain the variation in cancer risk, similar to that observed in HPVs (6). The EBV genome encodes close to 100 open reading frames, several of which are expressed consistently in human cancers [EBV nuclear antigen 1 (EBNA1); latent membrane protein 1 (LMP1)], and some of which have growth-transforming activity and are essential for EBV immortalization of B cells in vitro and tumorigenesis in animal models (LMP1, EBNA2, EBNA3C). Its mechanisms of viral subterfuge include encoding viral pirates of the B cell receptor, CD40-like co-receptors, and the Notch family of transcription regulators (7). The EBV genome also encodes over 20 microRNAs and other noncoding RNAs that are expressed at high amounts in human cancers and have tumorigenic properties, including the potential to be transmitted via exosomes to noninfected neighboring cells (8). EBV can adopt variant gene expression patterns that enhance its adaptability and help it to evade host immune recognition. EBV latent infection can also epigenetically suppress host tumor suppressor genes, providing a potential “hit and run” mechanism for viral oncogenesis (9).

Although there is a vaccine for HPV (10), none yet exists for EBV. At least one effort to develop a vaccine targeting the EBV glycoprotein gp350 was effective in reducing the incidence of infectious mononucleosis (11), but did not prevent the occurrence of latent infection, raising concern that the vaccine would not prevent most EBV-associated cancer. B cell lymphomas arising from EBV have been successfully treated by adoptive immunotherapy (12), but this approach has proven labor intensive and technologically challenging. Thus far, there are no selective treatments for EBV-associated disease, although efforts are under way to develop both biological and pharmacological inhibitors of viral proteins and oncogenes. And as more diseases with potential links to EBV infection are revealed, such as multiple sclerosis and lupus erythematosus (13), the need

for personalized therapies for treating EBV will continue to grow.

To date, EBV is estimated to be responsible for ~200,000 cancers worldwide (4). The U.S. National Institutes of Health (NIH) recently called for a new initiative to reduce global cancer incidence, with EBV among the top candidates for future advances (14). Further clinical testing of the gp350 vaccine, as well as development of second-generation vaccines and diagnostics to measure vaccine efficacy and cancer risk factors, have been recommended by an NIH-sponsored panel (14, 15). Among the new generation of vaccines will be those that treat latently infected individuals with existing EBV-driven cancers as well as those that are at high risk for develop-

ing EBV-associated disease (e.g., solid organ transplant recipients). Any vaccine that stimulates a strong and selective T cell response to EBV-positive tumor cells is likely to provide protection and therapeutic benefit. It is to be hoped that the successes of HPV and hepatitis B virus vaccination programs will encourage new and ongoing efforts to find a suitable immunological or pharmacological treatment for EBV and associated disease. An efficacious antiviral would also provide the final confirmation that EBV is indeed a tumor-causing virus.

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PHYSICS

Flow of Control in Networks

Jukka-Pekka "JP" Onnela

Many complex systems can be viewed as networks, in which nodes represent system elements and edges correspond to interactions between those elements. In such networks, a subset of nodes—the driver nodes—can yield control of the entire network when they are driven by external signals (1–3). However, to control a system, one must know not only what parts need to be controlled but also why these particular parts need to be controlled. On page 1373 of this issue, Ruths and Ruths (4) put forward an elegant framework that elucidates the origin of control in networks. The framework divides nodes into three categories based on how they affect the flow of control in networks. This approach gives rise to control profiles that the authors use to classify a host of empirical and synthetic networks. The results suggest that networks from different domains but in the same category may be more similar to one another than previously thought.

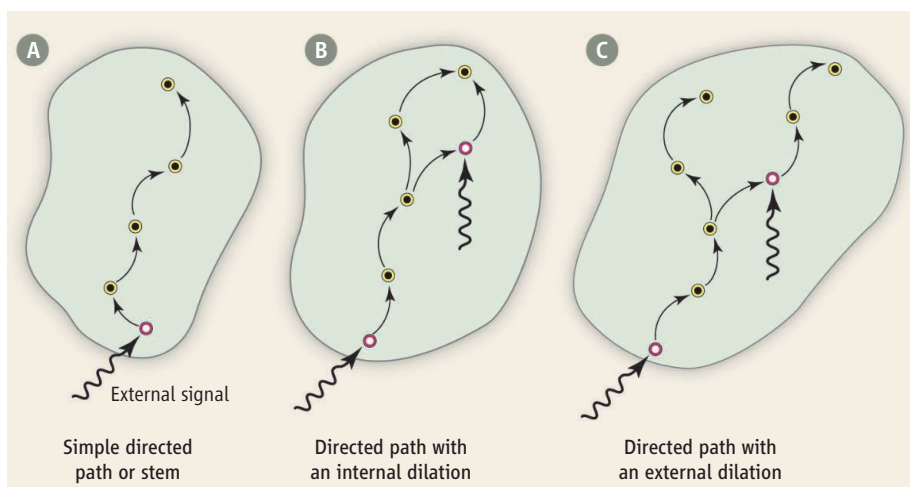
To understand the idea of network control, consider a dynamical process that unfolds on a network, such as an airport transportation network, where the state of a node encodes the number of passengers at that node. If a directed edge leads from node A to node B, then the state of node A influences the state of node B. The system is controllable if it can be driven from an arbitrary initial state to a specified final state in a finite time. In the absence

of loops and cycles, each node can control at most one of its neighbors (5). Together, these node-to-neighbor couplings give rise to disjoint directed paths of control in the network; each path, or stem, needs its own independent control. If we can identify the stems, we can control the network.

The standard way to find the set of controllable nodes is to use the maximum matching algorithm (6), where “maximum match-

A simple framework allows the classification of complex networks based on the flow of control.

ing” refers to the maximum set of edges that do not share start or end nodes (1). A node is matched if an edge in the maximum matching points to it; otherwise, it is unmatched and needs its own independent control. If all stems were simple directed paths (see the figure, panel A), the number of independently controlled nodes would equal the number of source nodes, which are nodes with no incoming edges.



Control-inducing structures in networks. In a simple directed path or stem (A), only the source node needs to be controlled. If a stem branches, either internal dilations (B) or external dilations (C) are introduced, both of which increase the number of nodes that need independent control. This is because each node can control at most one of its neighbors and at a dilation a node has more than one neighbor. In panel C, the number of nodes that need independent control coincides with the number of sink nodes (nodes with no outgoing edges); this is not the case in panel B. Ruths and Ruths show that each control-inducing structure can be classified as a source node, an internal dilation, or an external dilation, and that the control profile of a network is the relative frequency with which each type occurs in the network.

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When a stem branches into two or more paths, a dilation occurs, increasing the number of nodes that need to be controlled. Ruths and Ruths distinguish between two types of dilations. In an internal dilation, a path splits into two paths that soon rejoin, much like two lanes of traffic that momentarily split only to merge again soon after (see the figure, panel B). In an external dilation, a path splits without subsequently rejoining, like entering a T-intersection with two dead end streets (see the figure, panel C). An internal dilation does not increase the number of sink nodes, whereas an external one does, by one for each branching.

In the absence of internal dilations, only source nodes and external dilations need to be controlled. When internal dilations are present, the sum of the source nodes and surplus sink nodes gives a lower bound on the number of nodes that need control. In most networks, the number of internal dilations is relatively small, and the bound obtained as the sum of source and surplus sink nodes is therefore fairly tight.

Previous studies have categorized nodes and edges in control structures by considering whether a node always, never, or sometimes needs its independent control (3) and by investigating whether an edge is always, never, or sometimes part of the control structure (1). The degree distribution of a network (that is, the distribution of the number of incoming and outgoing edges of each node) correlates strongly with the number of required independent controls (1). Ruths and Ruths now show that the special role of source and sink nodes in control structures leads to a simple causal link between degree sequence and the size of the driver node set.

Based on the three types of structures that induce the need for independent control (see the figure), real-world and synthetic networks can be profiled by designating all nodes that need to be controlled into source nodes, internal dilations, and external dilations. The control profile of a given network is simply the fraction of nodes in each of these three categories. By investigating a range of directed real-world and model networks, Ruths and Ruths find evidence for three categories of networks, which they call source-dominated, internal-dilation-dominated, and external-dilation-dominated networks.

The authors argue that source-dominated networks, such as neural and social networks, allow relatively uncorrelated behavior across their agents and are thus suitable to distributed processing. Internal-dilation-dominated networks, such as food networks and airport interconnectivity networks, are mostly closed

systems and obey some type of conservation laws. Finally, external-dilation-dominated networks with their surplus sink nodes yield correlated behaviors across their agents that are downstream from a common source, as exemplified by trust hierarchies and transcriptional systems.

The framework appears to assume that there are no intrinsic nodal dynamics. If self-loops (directed edges that connect a node to itself) were present at each node, such that the future state of a node depended on its own current state, all nodes could be driven by a single external control, given that loops and cycles are self-regulating structures (7). However, as the authors point out, if the time scale of interactions with network neighbors is much faster than that of the intrinsic dynamics, it is reasonable not to consider intrinsic nodal dynamics, especially for making comparisons across different types of networks.

Some studied networks will contain false positives and false negatives: The network representation of the system as obtained from empirical data will contain some edges that are not present in the real system, and will not contain some other edges that are present in the real system. Ruths and Ruths' framework may be particularly sensitive to false positives and false negatives, because they could change the classification of nodes in a non-

trivial manner. Furthermore, the data collection processes that introduce false positives and false negatives may be different across different network types. The impact of false negatives could be investigated by subsampling observed networks to determine how sensitive the control profile of a given network and its subsequent classification are to different forms of sampling.

The framework put forward by Ruths and Ruths is elegant in its simplicity and helps us to understand and interpret previous findings. It also points to interesting avenues for future work, such as the development of more realistic models of directed networks in the context of network control. Going forward, the framework provides a useful tool for moving from passively observing networked systems to pursuing control over them.

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DEVELOPMENT

Counting the Ways to Decode Dynamic Signals

Roy Wollman

The social amoeba *Dictyostelium discoideum* decodes an oscillatory signal to measure time.

The role of biological signaling networks is to reliably transmit specific information about the extracellular environment to multiple intracellular downstream effectors, allowing the cell to adjust its physiological state to changing conditions. One mechanism that cells use to enhance the performance of signaling networks is the temporal modulation, or dynamics, of the transmitted signals (1–4). The key role that modulating temporal activity of the signal plays in information transmission makes

signaling dynamics an attractive target for therapeutic approaches that interfere with the transmission of specific types of information through the network (5). The diversity of temporal modulation strategies seen in various signaling networks suggests that there is no single optimal strategy for making use of dynamic information. Therefore, to uncover the benefits of temporal modulation strategies, it is important to understand how the suitability of each type of signaling dynamics is matched to the nature of the particular information that is being transmitted. Some types of information are transmitted through frequency-modulated signals, whereas other types are transmitted through modulation of signal amplitude or duration.

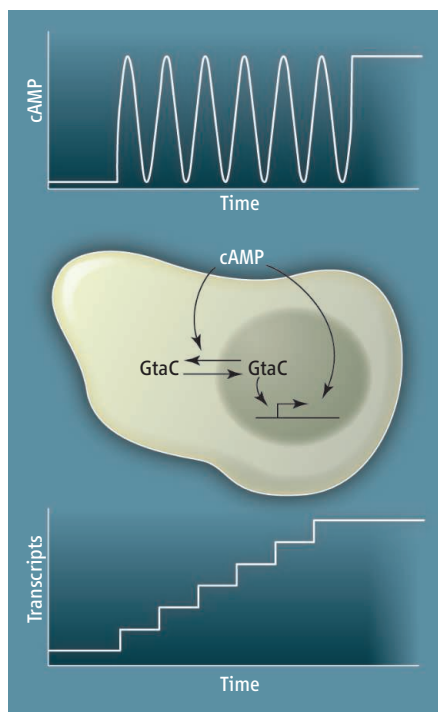
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Work by Cai *et al.* (6) on page 1329 of this issue identifies how the social amoeba *Dictyostelium discoideum* decodes a temporally dynamic signal to coordinate its development in response to starvation.

In *D. discoideum*, starvation triggers the chemotactic aggregation of tens of thousands of cells that then coordinate their activities to develop into a multicellular slug, collectively migrate, and form a fruiting body. Such coordinated action of a large number of cells requires reliable communication about gene expression and developmental state. Failure to communicate could cause a subset of the cells to develop at a different pace, resulting in their subsequent developmental arrest and death. Therefore, synchronous and successful development requires a common developmental timer shared by all the cells in the population. Research over the past 40 years has uncovered a complex synchronization mechanism that is controlled by periodic waves of cyclic adenosine monophosphate (cAMP) secreted by the amoebae and propagating throughout the population (7). Pulses of cAMP are necessary for the completion of the developmental program, which suggests that information about the developmental state of cells in the aggregate is encoded in the pulsatile dynamics of extracellular cAMP. However, the mechanism by which this developmental information is decoded by cells has been unknown until now.

Cai *et al.* found that the key signaling circuit that decodes the dynamic cAMP signals in *D. discoideum* involves GtaC, a GATA family transcription factor. GtaC is negatively regulated by cAMP: An increase in the concentration of extracellular cAMP results in the glycogen synthase kinase 3 (GSK3)–dependent export of GtaC from the nucleus to the cytoplasm. Tracking mRNA expression at the single-cell level demonstrated that there is a delay between the cAMP pulse and the resulting decrease in expression of genes positively regulated by GtaC. Because cAMP is known as an activator of gene expression during *D. discoideum* development, its new regulatory role as a delayed inhibitor of gene expression suggested that the circuit that decodes cAMP acts as an incoherent feedforward module. In an incoherent feedforward module, an upstream activator regulates a downstream target through two opposing arms: a fast activator and a slow inhibitor. The delay between the two regulatory arms results in a pulse of target activity.

The identified circuit carries out two key functions: It acts as a low-pass filter and as a counter. The low-pass filter enables the cell to filter out high-frequency cAMP pulses.



Decoding temporal signals. During starvation-induced aggregation in *D. discoideum*, extracellular concentrations of cAMP oscillate. A decoding mechanism of these oscillations that uses the translocation of a GATA transcription factor between the cytoplasm and the nucleus allows cells to count the number of pulses and synchronize the transcriptional programs across the population.

Because the generation of pulses involves a positive feedback mechanism, the system is prone to be noisy, and the ability to be insensitive to short (less than 6 min), high-frequency pulses will increase the robustness of the transition into collective behavior (8). The second function of this circuit is its ability to transform each pulse of cAMP longer than 6 min into a single pulse of transcriptional activity. Therefore, the expression of GtaC-dependent genes will increase approximately linearly as a function of the number of permitted pulses rather than the overall concentration of cAMP (see the figure). By manipulating extracellular cAMP levels, Cai *et al.* demonstrated that the expression of key developmental genes increases with the number of cAMP pulses, supporting the interpretation that cells count the number of pulses to which they are exposed.

The invention of the pendulum clock in 1659 was a technological breakthrough because it allowed the measurement of time with unprecedented accuracy, with an error of approximately 1 min per day (9). A pendulum clock translates an oscillatory input into a time measurement by decoding each cycle as a single action. This transformation

of an oscillatory signal into a count was the key to the incredible accuracy of pendulum clocks relative to previous timekeeping technologies. The circuit identified by Cai *et al.*, similar to the oscillations of the pendulum clock, allows each cell in the collective to translate pulses of cAMP into temporal information. The accuracy of pulse counting as a timekeeping mechanism makes oscillatory encoding a temporal modulation strategy that is adapted to the synchronization challenge faced by *D. discoideum*.

Pulsatile gene expression has now been identified in several experimental systems and has been shown to provide various benefits to cells (10). The encoding of oscillations of cAMP through a population-level signaling circuit that is then decoded by single cells through an incoherent feedforward loop provides a mechanism that can explain how a collection of tens of thousands of cells can synchronize their developmental states. The use of oscillatory dynamics has the potential to facilitate accurate timekeeping by cells in the population, enabling a synchronized transition from a unicellular to a multicellular developmental program.

Signaling networks encode information about events in the extracellular environment into specific signaling dynamics that are then converted (i.e., decoded) into specific actions important for cell physiology (11). The work of Cai *et al.* shows that the coupling of population-level temporal encoding of oscillatory dynamics with single-cell decoding of these pulses into temporal information can act as an accurate developmental timer and thereby provide an important selective advantage for *D. discoideum*. Future studies of signaling dynamics may uncover additional system-specific benefits and costs entailed in the use of various encoding and decoding strategies to transmit biological information through signal transduction networks.

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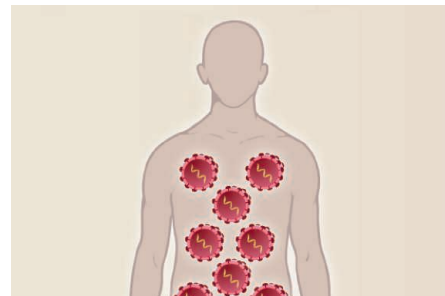


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HIV genetics and heritability of virulence likely contribute to disease severity despite intense within-host selection.

Virulence and Pathogenesis of HIV-1 Infection: An Evolutionary Perspective

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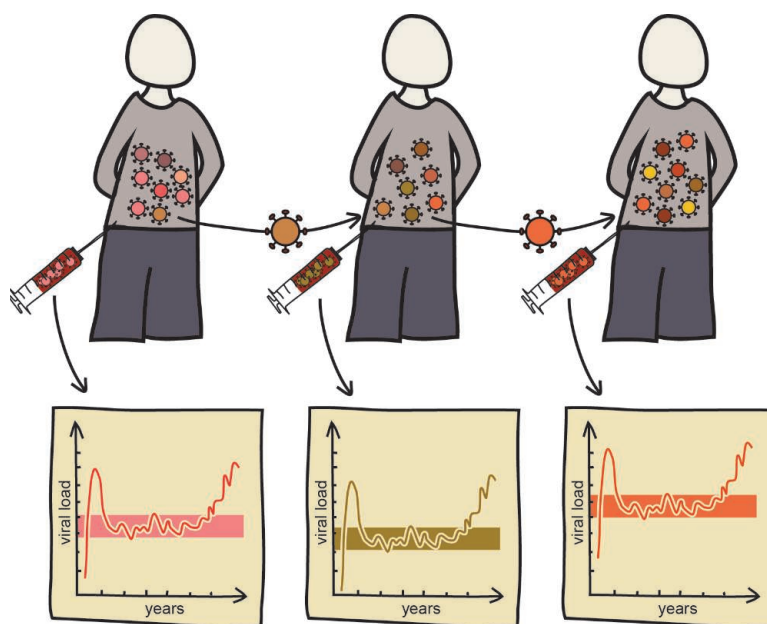


Background: Why some individuals develop AIDS rapidly whereas others remain healthy without treatment for many years remains a central question of HIV research. Of the quantities that predict how quickly an untreated infection progresses, the most widely used is set-point viral load. This measure varies by orders of magnitude between infected individuals and is predictive of infectiousness and time to onset of AIDS. Host factors, predominantly linked to the immune system, are known to influence the set point, but much variation remains unexplained.

Advances: We review recent evidence showing that HIV genotype influences the set point viral load far more than anticipated. Our summary of published estimates suggests that 33% (95% confidence interval, 20 to 46%) of the variation is attributable to the virus. Because set-point viral load is heritable (partially controlled by virus genotype) and is linked to transmissibility, it is likely to have evolved to maintain transmission fitness and may continue to evolve in response to diverse selection pressures. These findings are unexpected and paradoxical because rapid and error-prone viral replication should favor within-host adaptation and rapidly scramble signals of viral genotype as infection progresses, rather than leaving a lasting footprint that is preserved throughout an infection and from one infection to the next in transmission chains.

Outlook: We propose that resolving the paradox of heritability of set-point viral load will provide new insights into the mechanisms of HIV pathogenesis. To this end, we provide three parsimonious, testable, and nonexclusive explanatory mechanisms. The first states that HIV evolution in virulence genes is more functionally constrained than previously thought. The second proposes that virulence of HIV is mediated through the virus's capacity to systemically activate target cells in which it can efficiently replicate. The capacity to activate would not be expected to evolve rapidly because it does not provide a specific selective advantage to virus strains that activate more cells; rather, it is an advantage shared by all viruses. The third mechanism implicates the preferential transmission of viruses that are stored in nonreplicating cells or during early infection, and the disproportionate influence on long-term pathogenesis of these early viruses.

In addition to these insights into mechanisms of pathogenesis, we believe that this research highlights a major gap in our knowledge of HIV. The identification of the genetic determinants of HIV virulence, which appear to vary between closely related strains of the virus, should be a major priority. Thus, whole-genome association studies that are focused on the virus genome should



A transmission chain with heritable virulence. Individuals infected with HIV show differences in clinical progression. Untreated infections are characterized by viral loads (the viral particle density in the blood) that are relatively stable for years, but they can differ by orders of magnitude between individuals. Host factors clearly influence viral load, but viral loads have also recently been found to correlate among individuals in transmission pairs and chains. This indicates a moderate to strong influence of viral genotype on the viral load. Strikingly, this influence persists for years and across transmission events, despite intense within-host viral evolution.

be pursued and expanded, as well as more functional and mechanistic studies, which could be guided by hypotheses such as those presented here.

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Virulence and Pathogenesis of HIV-1 Infection: An Evolutionary Perspective

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Why some individuals develop AIDS rapidly whereas others remain healthy without treatment for many years remains a central question of HIV research. An evolutionary perspective reveals an apparent conflict between two levels of selection on the virus. On the one hand, there is rapid evolution of the virus in the host, and on the other, new observations indicate the existence of virus factors that affect the virulence of infection whose influence persists over years in infected individuals and across transmission events. Here, we review recent evidence that shows that viral genetic factors play a larger role in modulating disease severity than anticipated. We propose conceptual models that reconcile adaptive evolution at both levels of selection. Evolutionary analysis provides new insight into HIV pathogenesis.

The past few years have seen impressive progress in HIV research, particularly in prevention, including the definition and characterization of broadly neutralizing antibodies for vaccine design (1), demonstration that antiretroviral treatment almost fully blocks transmission (2), and trials of preexposure prophylaxis that have met some success (3). Nevertheless, our understanding of pathogenesis—of why some patients progress to AIDS quickly in the absence of treatment, whereas others remain AIDS-free for many years—remains largely unknown (4). In this Review, we explore how evolutionary theory grants us a fresh perspective that leads to unexpected insights into HIV biology.

Viral load is the density of virus in someone's blood and by proxy in the rest of their body (5) and is relatively stable during asymptomatic infection, fluctuating around a value known as set-point viral load (SPVL) (Fig. 1A). A particularly important task in HIV pathogenesis research is to explain the orders-of-magnitude variation in SPVL. SPVL ranges from as low as <20 viruses per milliliter of blood plasma up to 10⁶ (Fig. 1B) (5–7) and correlates with virulence and infectiousness (Fig. 1C). Of the surrogate markers of infection severity, or virulence, SPVL is the most robust and widely used (5). Virulence

is defined in untreated asymptomatic infection as the speed of progression from infection to AIDS, by which point CD4⁺ T cells are depleted, immunity is exhausted, and disease becomes apparent.

To date, most research on explaining variation in SPVL has focused on host factors, and particularly on components of the human immune response. Genome-wide association studies (GWASs) have identified more than 300 single-nucleotide polymorphisms (SNPs) in human lymphocyte antigen (HLA)-Class I genes (which implicate the T cell response), and none elsewhere (8, 9), thus confirming earlier findings in the area (10). Other non-SNP host-genetic modifiers of SPVL include the Δ 32 deletion in the CCR5 co-receptor (which reduces the ability of CCR5 tropic strains of the virus to infect cells) (11) and copy number variation in killer cell immunoglobulin-like receptor genes (which implicate the innate immune response) (12). Despite the groundbreaking nature of these studies, the total proportion of variance in SPVL explained by host factors is limited to ~13% (increasing to 22% when adding age and sex as explanatory variables) (8).

In parallel to this research, new data and analyses suggest that viral factors are as important as host factors, if not more so, in determining disease severity. Here, we review the evidence for the importance of viral factors, as quantified by heritability of SPVL (defined in Box 1 and below); discuss how an evolutionary perspective may help us identify these factors; and generate new insights into the mechanisms of HIV pathogenesis. We suggest that the influence of viral factors on SPVL, and consequently on disease severity, have frequently been underestimated.

Viral Virulence Factors

Heritability is defined as the proportion of variance in a phenotype that is attributable to the

variation in the underlying genotype (13). For HIV-1 SPVL, it is defined as the proportion of variance in SPVL that is attributable to viral genetic factors, which we term viral virulence factors because they influence the severity of untreated infection. For an infection trait such as SPVL, heritability is closely related to (but not equivalent to) the relationship between the trait value in the transmitter (donor) host and in the recipient host within a transmission pair. The higher the heritability of SPVL, the more SPVL will be similar between the individuals in the transmission pair.

There is currently a controversy in the field concerning the magnitude of heritability of SPVL (14–21). At the high end, Alizon *et al.* (2) reported heritability estimates exceeding 50%. At the low end, Yue *et al.* (19) stated that an “analysis of 195 transmission pairs from Lusaka, Zambia revealed that viral load in the transmitting source partner contributed only 2% of the variance in seroconverter early set-point viral load ($P = 0.046$ by univariable analysis).” We demonstrate in (22) that this variation of an order of magnitude (2 to 50%) between these estimates does not arise from major differences between patient cohorts or any computational issues, but rather because these studies are reporting different quantities that are only indirectly related to heritability. Using a consistent framework, heritability of SPVL in heterosexual couples in sub-Saharan Africa is estimated to be 33% (95% confidence interval, 20 to 46%) and is similar across studies (Box 1). These reinterpreted estimates are also broadly consistent with the results of phylogenetic methods to estimate heritability of SPVL (14, 15), although estimates obtained in this manner have been more dispersed.

Given the potential importance of viral genotype in determining SPVL, we find it surprising that we do not yet have a firm idea of what the virus virulence factors that affect SPVL are, the mechanisms by which they act, nor how they are preserved from one infection to the next despite potentially thousands of rounds of replication and ongoing evolution within the host. However, the two most likely candidates are viral genetic features that modulate the replicative capacity of the virus and those that influence its capacity to induce immune activation.

We consider the replicative capacity (RC) of the virus to be a likely correlate of viral virulence (23–27). RC is defined as the mean number of cells infected by a typical infected cell (directly or by viral production and infection) with plentiful access to target cells and in the absence of effective immune responses. In practice, although defined *in vivo*, it is usually measured in *in vitro* fitness assays in which by definition immune responses are absent. To study the clinical relevance of RC, Kouyos and colleagues predicted RCs from viral sequences based on a

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complex statistical model trained on many in vitro measurements and then demonstrated correlation with SPVL (24, 28).

Another study reported that the main feature distinguishing “elite controllers” (individuals who do not progress to AIDS despite decades without treatment) was that they were infected by very unfit viruses, with low RC (20). It appears that the low fitness of the initial infecting viruses was the only factor the elite controllers had in common. Mutations found in some of the low-fitness viruses included drug-resistance mutations as well as escape mutations from HLA-B57 and homologs (HLA-B57 is one of the human genotypes most consistently associated with slow progression to AIDS). Escape mutations with low RC are likely to evolve within a host when the selection pressure exerted by the immune system is stronger than intrinsic selection for increasing RC. However, only 4 out of 20 elite controllers in (26) carried HLA-B57 genes or homologs, whereas 6 out of 20 acquired HLA-B57 escape mu-

tations from their infectors. Another study showed that viral escape mutations from protective HLA reduce SPVL, not just in the patient carrying the protective HLA but also in those who are infected by these individuals and who do not harbor that particular HLA type (29). Even when these unfit viruses acquired compensatory mutations that increase RC during the course of an infection, an increase in viral load was not always observed (26). This latter finding, that later reversion of costly mutations present in early infection does not lead to increased viral load, can be explained if events taking place early in an infection are disproportionately important determinants of SPVL—that is, if it is initial infection with an unfit low RC virus, rather than maintaining this viral genotype during the whole course of infection, that results in a low SPVL.

The second category of viral virulence determinants that might influence viral load are factors inducing CD4⁺ T cell activation because activation of CD4 cells is a prerequisite for rendering

cells permissive to infection (30, 31). Most prominently, *nef* (32) but also *vpr* (33), *tat* (34), and *env* (35) have been implicated in modulating T cell activation. Because CD4⁺ T cell activation correlates with viral load (36), these viral genes may have an effect on viral load.

Trade-Offs and Population-Level Evolution of Virulence

Not only is viral load the most widely used prognostic indicator of disease progression, but it is also positively correlated with infectiousness (Fig. 1C) (7). This results in an evolutionary trade-off for the virus so that if there is a low SPVL, there will be more time for onward transmission before the host dies, but the probability of transmission per contact will be low. If a higher SPVL establishes, then there will be less time for onward transmission, but the probability of transmission per contact will be higher. In the face of such a trade-off, natural selection should favor pathogen strains that maximize the

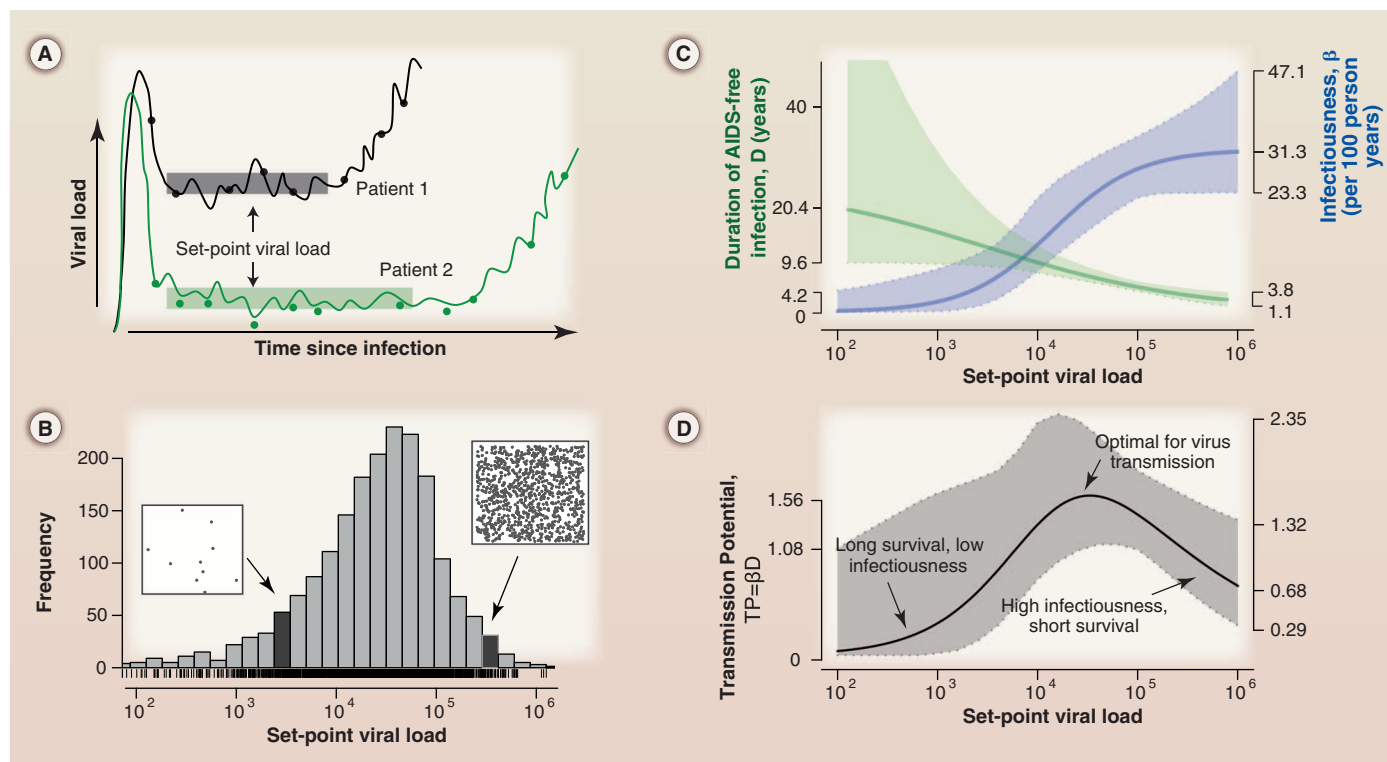


Fig. 1. The evolutionary epidemiology of viral load. (A) The typical pattern of viral load in untreated HIV-1 infection, with a very high peak during the first weeks of infection and a gradual increase in the late stages of infection. During asymptomatic infection, viral loads are often relatively steady, fluctuating around the SPVL. (B) The distribution of SPVL in a cohort of patients infected with subtype B virus. The distribution is similar across populations and across viral subtypes (70). The log-scale is shown on the x axis, which demonstrates variability over orders of magnitude. To illustrate the extraordinary extent of this variability, shown in the inset plots are simulated viral density in 5 μ L of peripheral blood for two values at either side of the distribution of viral load; thus, viral load is a strong predictor of both infectiousness and rate of progression to disease. The distribution of SPVL was previously described with (7), and we have updated this to include SPVL data from 2015 seroconverters in the cohort (with kind permission from de Wolf and Reiss). (C) Infectiousness,

estimated within heterosexual couples, is shown in blue. The severity of infection, estimated as the mean time from seroconversion to AIDS, is shown in green [from (7)]. Lines show best fit, and filled areas show 95% confidence intervals. (D) The transmission potential is a summary measure of the epidemiological “success” of an infection with given SPVL: It is the mean number of expected secondary infections over the whole chronic and asymptomatic infectious life span, estimated as the product of infectiousness and duration of asymptomatic infection [from (C)]. The close agreement in between the optimal evolutionary strategy [labeled in (D)] and the distribution of viral loads [plotted in (B)] suggested the hypothesis that the distribution of viral loads is in fact the outcome of viral adaptation to maximize the transmission potential (7). None of the calculations account for the effect of treatment. This is reasonable for an evolutionary analysis because treatment has unfortunately only become widely available in recent years.

number of onward transmissions per infection—the basic reproduction number R_0 (7, 37–39).

If, as we have argued, HIV virulence factors exist, if these are heritable and are partially transmitted from one infected person to the next, and if in addition these factors affect viral fitness at the between-host level, then a natural corollary is that HIV virulence should be subject to natural selection at the population level. If strains differ in virulence, and this translates to differences in R_0 , then evolution will tend to select strains with the highest R_0 .

In 2007, Fraser *et al.* (7) proposed that HIV-1 SPVL is a life-history trait that has evolved to maximize the number of transmission events. This is quantified by the transmission potential

(Fig. 1D), which is the number of people that one infected individual with a given SPVL might infect on average during their asymptomatic lifespan. Crucially, the distribution of SPVLs is clustered around values that maximize the transmission potential (Fig. 1, B and D), suggesting that the virus has evolved to maximize its transmission potential in untreated infection. Repeating the analysis with R_0 rather than transmission potential is more complicated because estimation of R_0 requires assumptions about the distribution of SPVL generated by a strain and the dynamics of the sexual network, and should include transmission during early and late stages of infection in addition to chronic asymptomatic infection. However, such a calcu-

lation leads to the same conclusion, that HIV has evolved to maximize the number of onward infections (7).

Conceptual Models Reconciling HIV Adaptation at Multiple Levels

Within-host evolution of HIV is a rapid and dominant part of the viral life cycle (Fig. 2A). The virus can complete a full round of replication in an infected host in less than 2 days (40, 41), is under constant immune selection, and can evolve drug resistance in weeks (41). In view of ample evidence for high turnover and rapid viral evolution in the host, the finding reviewed in Box 1—that viral influence over SPVL may be partially preserved from one infection

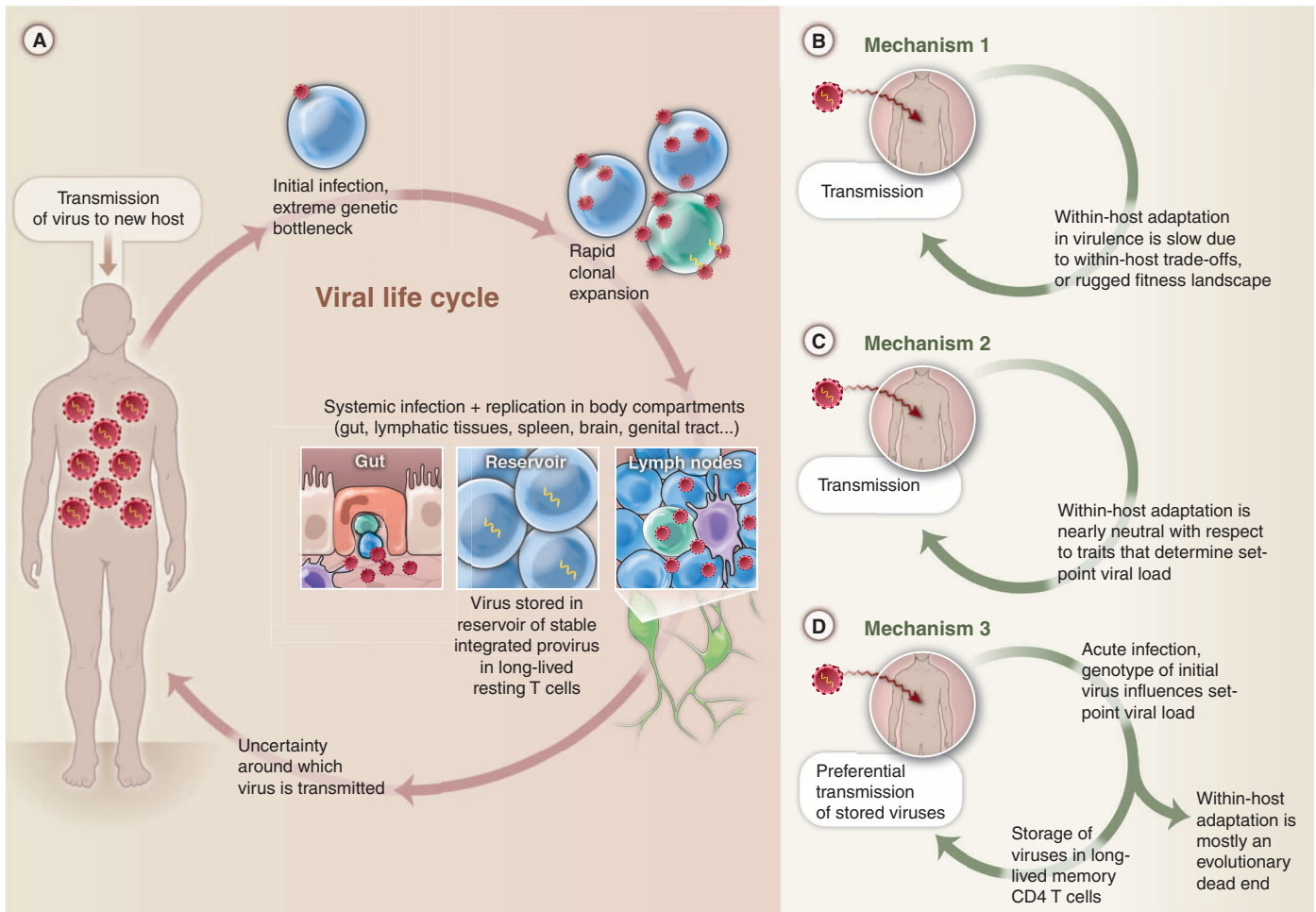


Fig. 2. Mechanisms that could reconcile rapid within-host evolution with heritability of SPVL and adaptation to maximize transmission opportunities. (A) Illustration of a full transmission-to-transmission viral replication cycle. During or shortly after transmission, the virus experiences a very strong bottleneck (71) and expands clonally, mostly in activated CD4⁺ T cells. Most viral replication is very fast, with a cellular life cycle of 1 or 2 days (40, 41), but a reservoir of virus in long-lived CD4 cells is quickly established, is continuously replenished, and persists for life (52, 53). Viral replication quickly becomes systemic, assisted by chronic and persistent immune activation, with gut-associated lymphatic tissue and germinal centers in other lymphatic tissues being particularly privileged sites of replication (53, 59). The most uncertainty in the life cycle surrounds which viruses, if any, are preferentially transmitted to found new infections in other hosts. (B to D) Three mechanisms that could reconcile within-host adaptation with heritability and

population-level viral evolution to maximize transmission. These mechanisms are described by schematic representations of the viral life cycle. (B) In mechanism 1, population-level evolution is explained by evolutionary constraints that slow within-host evolution of virulence traits in the host, perhaps owing to virulence genotypes requiring complex combinations of mutations rather than individual noninteracting point mutations. (C) In mechanism 2, population-level evolution is explained by an absence of selection within the host for viral virulence factors. (D) In mechanism 3, population-level evolution is explained by separation of within-host and between-host adaptation caused by preferential transmission of viruses that are stored during early infection, together with disproportionate influence of the founder genotype on SPVL.

Box 1. Heritability quantifies the effect of viral genotype on SPVL.

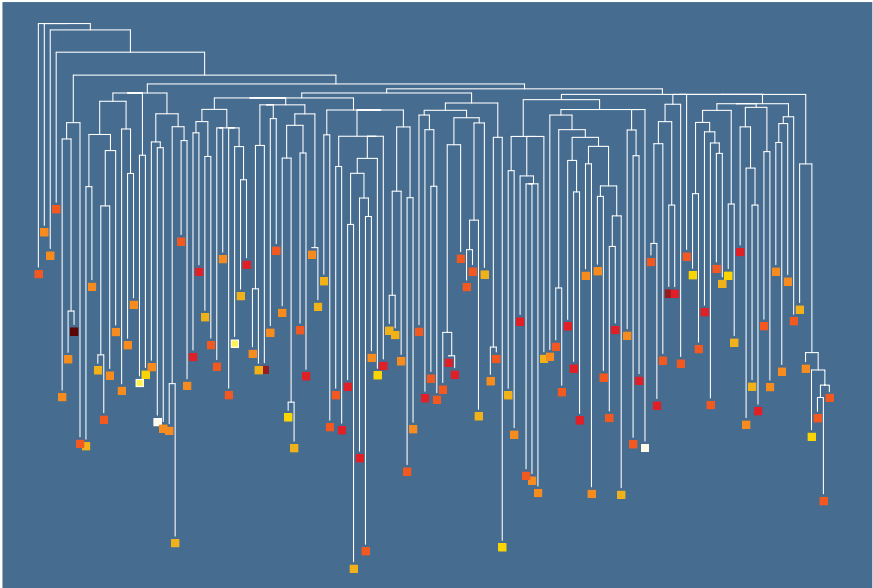
In quantitative genetics, the heritability of a trait is the proportion of the variance in the phenotypic value observed in a population that is attributable to genotype (13). This measure indicates the importance of genetic variation in shaping phenotypic variation. Recently, this concept has been applied to infectious diseases: For HIV, the heritability of a phenotypic trait (such as virus load) is the proportion of variance in this trait that is attributable to viral genetic factors. Because SPVL is a feature of the natural history as a whole, heritability of SPVL is defined with respect to the ensemble of genotypes of viruses that infect and evolve within the host. The simplest study design to measure heritability in SPVL is to look at the similarity in trait values between individuals in couples in which transmission is strongly suspected to have occurred (16–21). If SPVL is indeed heritable from one infection to the next, there should be similarity between a “recipient” and “donor” viral load. More technically, broad-sense heritability (conventionally denoted h^2) is estimated by the slope of the regression line (r) of the recipient viral load on the donor viral load (supplementary ma-

terials). Estimates of r obtained by independent studies have been quite consistent, despite differences in their presentation due to differences of focus: Few studies have presented h^2 as the key summary statistic. Estimates of h^2 , adjusted for many confounders, are shown for several studies in Table 1. An alternative design for estimating heritability, inspired by the phylogenetic comparative method, is to use virus sequence data from each host to reconstruct a phylogeny of infections and estimate the relationship between virus genetic relatedness and SPVL (Fig. 3). On the basis of this approach, Alizon *et al.* (15) found that up to 59% of variance could be explained by viral genotype. Variation among heritability estimates obtained in different studies should not necessarily be taken as an expression of the uncertainty of the individual estimates because heritability is a measure that also depends on the population in which it is estimated (13). For example, a very low-diversity epidemic resulting from an explosive burst of transmissions would exhibit lower heritability than would a high-diversity mature epidemic because of low background diversity in genotypes against which to make comparisons.

N couples	Heritability	Study (reference)	Country, subtype(s)	Adjustments
97	36% (6 to 66%)	Hollingsworth <i>et al.</i> (17)	Uganda, mostly A, D, and recombinants	Age, sex, subtype, symptomatic genital ulcer disease (GUD)
141	44% (19 to 69%)	Lingappa <i>et al.</i> (18)	Partners in Prevention (14 sites in East and Southern Africa), diverse subtypes	Age, sex, subtype, sexually transmitted infection, GUD, circumcision, hormonal contraceptive use, source partner characteristics
195	26% (8 to 44%)	Yue <i>et al.</i> (19)	Zambia, mostly C	Age, sex, HLA, HLA sharing between partners
433	33% (20 to 46%)	Overall summary estimate (weighted by standard error)		

Table 1. Summary of estimates of heritability of SPVL from transmission couples in sub-Saharan Africa, and overall aggregate summary estimate. Studies reviewed here were selected as studies of transmission among heterosexual couples in sub-Saharan Africa.

Fig. 3. Phylogenetic proximity yields similar values for heritable traits. Shown is the phylogeny of HIV isolated from 134 Swiss men who have sex with men with well-defined SPVL (indicated by the color of tips). Heritability is estimated by comparing phylogenetic proximity to similarity in SPVL (15).



to the next—is unexpected. Evolution to maximize population-level transmission potential is even more surprising. From an evolutionary perspective, we would naively expect evolution to induce adaptation at the level most proximal to viral replication: within the host, a process sometimes termed “short-sighted” evolution (42, 43). We might expect most adaptation to be targeted at optimal replication in local tissues, with no correlation of viral loads between transmitting individuals, rather than the adaptation to maximize the transmission potential of the virus suggested in Fig. 1. Nonetheless, there is now sufficient empirical evidence that HIV heritability needs to be explained, and therefore that mechanisms of HIV pathogenesis need to be compatible with multilevel selection, allowing the virus to adapt to both proximal within-host and distant between-host selection pressures. This compatibility requirement is a strong constraint. We propose three possible (and not mutually exclusive) mechanisms which meet this constraint in Fig. 2, B to D.

Mechanism 1: Slow Evolution of Replicative Capacity

We know that viral turnover and mutation rate are both high, but this does not necessarily imply that all viral phenotypes, such as virulence traits, should evolve rapidly (Fig. 2B). First, the within-host fitness landscape may be highly complex and rugged (28, 44), which might slow within-host evolution and adaptation. Second, viral strains with a high *in vitro* replicative capacity might not reach sustained high frequencies within an individual if they are intrinsically more immunogenic, or because they are preferentially targeted by acquired immunity as a result of their dominance during early infection. For example, one common consequence of immune selection is the emergence of mutations that allow the virus to escape cytotoxic T-lymphocyte (CTL) responses. Many CTL escape mutations reduce the *in vitro* replicative capacity of the virus but are nonetheless under intense positive selection during the course of infection because they help the virus evade the host immune response (45). Compensatory mutations can restore fitness after escape, but evolution through a process of continuous immune escape and compensatory evolution may slow the overall pace of evolution of net replicative fitness and would shift the balance of selection toward population-level selection.

Mechanism 2: Little Within-Host Selection for Viral Load

It is conceivable that there are viral factors that strongly affect viral load but are neutral with respect to within-host fitness and thus do not evolve rapidly within a host (Fig. 2C) (46). Owing to the absence of strong within-host selection, such factors would result in the heritability of virulence and the evolution of viral loads that maximize the between-host transmission potential. Using a generic model of HIV replication,

Bonhoeffer *et al.* (6) proposed that differences in the rate of activation of target cells may be a major source of variation in virus load. Although it can replicate in many cell types, HIV replicates most efficiently in activated CD4⁺ T cells (31). If the virus activates target cells systematically, then any viral strain that activates cells provides a replication benefit that is not exclusive to just this strain but rather to all competing strains in the body, to the extent that all are provided with additional target cells. Thus, virus-induced activation of target cells can be regarded as contributing toward a “public good” (46), and there would be no selective advantage for a viral strain that increases the production of target cells. Which viral factor fulfills the required criteria for virus-induced and systemic target cell activation remains unclear. However, *nef* is clearly an interesting candidate. First, one of the *nef*-linked pathways of T cell activation may indeed be systemic because it is mediated through the release of soluble factors secreted by *nef*-expressing macrophages (32, 47). Second, patients with *nef*-deficient virus have low viral load (48, 49).

Mechanism 3: Influence and Transmission of Founder Viral Strains

Another explanation for heritability of SPVL and evolution of HIV to maximize transmission could be preferential transmission of viruses that have not undergone many rounds of replication in the host, either because transmission occurs during early infection (50) and/or because of the transmission of viruses that have been sequestered in long-lived cells [a process referred to as “store and retrieve” (51)]. This process, coupled with the disproportionate influence of the genotype of viruses from early infection on SPVL, would essentially make any within-host adaptation an evolutionary dead end for the virus (Fig. 2D). The viral response to multilevel selection would thus be akin to differentiation made between the germ line and soma, that could itself be an adaptive strategy of the virus to maintain transmissibility. During the course of HIV infection, a small fraction of infected CD4⁺ T cells transition into long-lived memory CD4⁺ cells containing an integrated copy of the viral genome, thus creating a long-lived reservoir of stored virus (52). Because much of this sequestration occurs during the early stages of infection (53) when viral loads are greatest, the reservoir acts as an archive of early viral sequences similar to the ancestral sequence (or sequences) that initiated the infection. Occasionally, sequestered virus is retrieved from the archive by the reactivation of a latently infected cell (52). If retrieved virus is preferentially transmitted, then the virus that an individual transmits will be similar to the virus that initiated the infection (Fig. 2D). There is evidence that viruses present during acute and early infection have a transmission advantage associated with distinct genotype (54) and phenotype (55),

lending support to this hypothesis. This mechanism also explains the long-standing phylogenetic puzzle of why HIV evolves faster within hosts than it does at the epidemiological level (51, 56, 57) and is partially supported by data from deep sequencing of viruses in transmission events (58). On the other hand, that both immune-escape and drug-resistance mutations are on occasion transmitted (26) demonstrates that on at least some occasions, it is extant and not stored viruses that are transmitted. The quantification of the preferential transmission of stored viruses requires further study.

To explain evolution to maximize transmission, it is not enough that a founder virus is preferentially transmitted. Its genotype also needs to influence viral loads throughout the course of infection more than variants that arise later. An example of such influence is that individuals infected with viruses with low RC tend to develop low SPVL even though the virus may increase its RC during the course of infection (26, 29), as previously discussed. One mechanism that could generate such an association would be if the RC of the virus affects the severity of acute infection and in particular the extent of damage to the gut. Gut damage would exacerbate the long-term level of microbial translocation, hence promote immune activation and increase the supply of target CD4⁺ T cells, and thus enhance the SPVL (59). Whatever the mechanistic basis, there is some empirical evidence that acute infection severity correlates with SPVL (60, 61).

Distinguishing Between the Mechanisms

Each of these mechanisms is sufficient to generate heritability of SPVL and allow population-level evolution to take place, but they are not mutually exclusive. Distinguishing which of these mechanisms are at work would require a better understanding of which aspects of viral biology influence SPVL. Large-scale HIV viral whole-genome association studies that search for correlations between viral polymorphisms and SPVL may enable us to detect which parts of the viral genome are correlated with disease severity, provided that the relevant mutations or genomic motifs are common and have sufficiently strong effect. Follow-up experimental work may elucidate their mechanism of action. In addition to recapitulating well-known findings on the association between human HLA and viral genotype and SPVL, Bartha *et al.* (62) reported a viral whole-genome association study on SPVL that did not find any statistically significant association between virus proteome and SPVL. The study, with *n* = 698 individuals, was however only powered to detect individual amino acid variants that explained at least 4% of variation of SPVL.

Future viral whole-genome association studies will require greater sample sizes and should include viral genetic polymorphisms and motifs in analyses. Identification of viral factors and of their mode of action will enable us to better

understand pathogenesis and to distinguish between viral replication as the primary factor influencing virulence, viral-induced immune activation, or other as yet unknown factors. Further testing to show whether ancestral sequences are indeed preferentially transmitted should also be an important goal of future research. This would require not only deep sequencing of viruses from donor and recipient patients close to the time of transmission, but sequencing from the donor sampled at multiple times before transmission. For ethical reasons, such studies would need to be retrospective and could also be expanded to consider transmission chains. Without these data, there is no way of determining whether a transmitted virus is an early variant that previously has been sequestered in long-lived cells. Studying viruses sampled from different body compartments in such transmission pairs would also be valuable because the blood compartment may be less relevant to sexual transmission than are the genital mucosa or semen, for example.

Beyond the Theory

Most recent research on HIV pathogenesis has focused on the host and its immune response, with more recent developments than can be summarized here [an up-to-date collection of reviews is available in (63), also from <http://perspectivesinmedicine.cshlp.org/cgi/collection/hiv>]. The aim of this Review has been to suggest the debate on HIV pathogenesis should include greater emphasis on the virus and its genotype, how viral variation interacts with the host response, and how certain heritable viral characteristics influence the course of disease. It is now difficult to ignore the mounting evidence that heritable viral virulence factors exist and that they have an important role in HIV pathogenesis. But what they are, how they are maintained during the course of infection and from one infection to the next—despite extensive viral replication between transmission events—and how they interact with now well-studied host factors are gaping holes in our knowledge. We have presented a framework to reconcile the potentially conflicting observations and key questions regarding HIV pathogenesis in the context of a synthesis based on evolutionary models.

In interpreting our findings, it is tempting to compare our estimate of HIV SPVL heritability (33%) with the lower estimate of 13% attributed to host genotype by GWASs, but these numbers are unfortunately not strictly comparable. GWASs tend to be conservative because of the problem of massive multihypothesis testing. In addition, they typically focus on “narrow-sense” heritability, which is the sum of individual contributions to SPVL directly attributable to single SNPs in human genomes (64). In contrast, the estimate of 33% for viral effects is “broad-sense” heritability and accounts for the total effect of viral genotype on SPVL, including possible complex interactions between viral SNPs and other genotypic motifs. As a result, broad-sense heritability

is always larger than narrow-sense heritability. Furthermore, if some of the variance is caused by interactions between host and virus genotype, there could be double counting in these measures. The relative role of host, virus, and interactions in explaining SPVL is still not defined, but we have argued that viral virulence factors that influence SPVL have often been underestimated.

Understanding viral virulence factors will help us to predict how the virus may evolve in response to different mass public health interventions. The widespread deployment of antiretroviral treatment, one of the great public health success stories of our time, alters the transmission trade-off and could plausibly select strains of increased virulence. SPVLs appear to have been increasing in some highly treated populations (65), although observations remain inconsistent (66). In predicting future changes, a further trade-off between increased virulence and drug resistance also needs to be considered because, as discussed above, drug resistance appears at least in some cases to attenuate the virus (26). If changing virulence is a concern, sentinel surveillance of viral load among treatment-naïve individuals could be straightforwardly combined with drug-resistance testing and would provide sufficient data. Furthermore, increasing virulence is expected to be slow and limited and does not challenge the current priorities of HIV treatment and prevention programs because it only reinforces the need for prompt diagnosis and treatment. Thinking further afield, an understanding of virulence factors could inform therapeutic strategies aimed at attenuation of infection in-host because at least in some cases of naturally attenuated infection, rapid reversion of virus back to higher levels does not seem to occur (26, 29, 49).

Our focus here has been predominantly on mechanistic evolutionary models of HIV virulence as assessed in individuals infected with closely related viruses. Future work could expand this approach to explain the documented difference between subtypes of HIV in which both disease and infectiousness may vary (67, 68), to explain virulence in the ancestral SIV viruses infecting a wide range of Old World monkeys and primates with very variable outcomes of infection (69), and to study virulence and multi-level selection in other RNA viruses causing chronic infection, such as human T lymphotropic virus and hepatitis C virus. Perhaps most importantly, an evolutionary perspective provides new insight into the basic mechanisms of HIV pathogenesis.

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Supplementary Materials
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 Materials and Methods
 Supplementary Text
 Figs. S1 and S2
 Table S1
 References (72–81)

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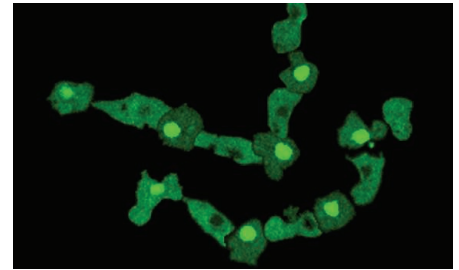


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The link between chemoattractant gradients, developmental signals, and gene expression in social amoebae is elucidated.

Nucleocytoplasmic Shuttling of a GATA Transcription Factor Functions as a Development Timer

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Introduction: Biological oscillations are universally found in nature and are critical at many levels of cellular organization. In the social amoeba *Dictyostelium discoideum*, starvation-triggered cell-cell aggregation and the early stages of developmental morphogenesis are orchestrated by periodic extracellular cAMP (3',5'-cyclic adenosine monophosphate) waves, which provide both chemotactic cues and developmental signals. Repeated occupancy of G protein-coupled cAMP receptors promotes optimal gene expression, whereas continuous stimulation suppresses the program. Although this activity was recognized nearly 40 years ago, the underlying mechanism for the stimulus-response pattern has not been elucidated.

Rationale: We reasoned that the mechanism decoding cyclic stimuli might be traced to a receptor-mediated regulation of a gene that is required for developmental progression. In a genetic screen for mutants that affect proper development, we discovered a GATA family transcription factor, GtaC, which is essential in this process. Furthermore, in different cells at different times, GtaC was found to either accumulate in the nucleus or disperse in the cytosol, suggesting that the dynamic cellular localization of GtaC could be a key to the relay of oscillatory signaling.

Results: We report here that GtaC exhibits rapid nucleocytoplasmic shuttling in response to periodic cAMP waves. Persistent

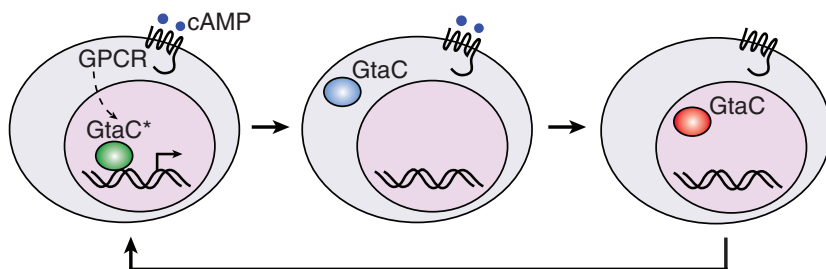
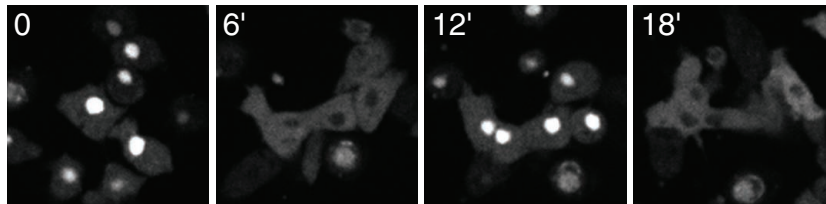
occupancy of the receptors by cAMP causes GtaC to translocate and remain out of the nucleus. This behavior requires a negative regulation of an intrinsic nuclear localization signal (NLS) by the N-terminal region of GtaC and

receptor-mediated phosphorylation by the glycogen synthase kinase GskA. When the exit of GtaC from the nucleus is inhibited by adding an exogenous NLS or by mutating the residues involved in phosphorylation, cAMP stimulation, either pulsatile or continuous, induces precocious gene expression and cell differentiation. The two apparently opposing effects of cAMP—it acts as a positive regulator of developmental gene expression but drives GtaC out of the nucleus—suggest that GtaC may be briefly activated during each stimulation cycle before it leaves the nucleus, while its return re-sensitizes the system. Consistently, we observe that each cAMP cycle generates a transient burst of GtaC-dependent transcription of the contact site A gene (*csaA*), a well-characterized developmental marker. We further demonstrate that this regulatory mechanism enables cells to modulate gene expression by counting low-frequency stimulations but filtering out high-

frequency signals; hence, the steady-state expression level of *csaA* is determined by the number of effective cAMP pulses experienced by the cell rather than the concentration of the cAMP stimuli or the overall time since the initiation of development. Computer simulations based on a corresponding logical circuit recapitulate GtaC-mediated accumulation of gene products under various stimulation regimes.

Conclusion: This work reveals a decoding mechanism by which oscillatory signals are used to guide gene expression and promote timely development. Tuning transcription to the number rather than the level of the external

GFP-GtaC



GtaC shuttling modulates developmental gene expression. (Top) Periodic nuclear enrichment of GtaC at times indicated in response to cAMP waves. **(Bottom)** In each cAMP stimulation cycle, GtaC transits through successive states of nuclear active (green), cytosolic (blue), and nuclear inactive (red), promoting transcription only in the active state. Repeated stimuli lead to reoccurring activations and incremental accumulation of gene products. The mechanism enables cells to count effective stimuli and modulate gene expression accordingly.

stimuli allows large populations of cells over an expanded territory to be developmentally synchronized. Similar mechanisms may operate in other circumstances where cellular plasticity is linked to repeated experience.

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Nucleocytoplasmic Shuttling of a GATA Transcription Factor Functions as a Development Timer

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Biological oscillations are observed at many levels of cellular organization. In the social amoeba *Dictyostelium discoideum*, starvation-triggered multicellular development is organized by periodic cyclic adenosine 3',5'-monophosphate (cAMP) waves, which provide both chemoattractant gradients and developmental signals. We report that GtaC, a GATA transcription factor, exhibits rapid nucleocytoplasmic shuttling in response to cAMP waves. This behavior requires coordinated action of a nuclear localization signal and reversible G protein (heterotrimeric guanine nucleotide-binding protein)-coupled receptor-mediated phosphorylation. Although both are required for developmental gene expression, receptor occupancy promotes nuclear exit of GtaC, which leads to a transient burst of transcription at each cAMP cycle. We demonstrate that this biological circuit filters out high-frequency signals and counts those admitted, thereby enabling cells to modulate gene expression according to the dynamic pattern of the external stimuli.

Rhythmic phenomena play a critical role in many cellular processes, such as the cell cycle, the circadian clock, the activity of neuronal and cardiac cells, the glycolytic system, and the segmentation of somites. In many cases, the spatiotemporal pattern is as important as the cellular activity itself. For example, the frequency of delivery rather than the total amount governs the physiological efficacy of certain hormones, oscillatory rather than persistent expression of regulatory genes is required for the maintenance of neural progenitor cells, and spaced rather than massed training trials are more reliable at inducing long-term facilitation (1–3).

A fascinating example of biological oscillations occurs in the social amoeba *Dictyostelium discoideum*. In this organism, starvation-triggered chemotactic aggregation and multicellular development are mediated by periodic extracellular cAMP (cyclic adenosine 3',5'-monophosphate) signals (4, 5). Through the coordinated action of a G protein (heterotrimeric guanine nucleotide-binding protein)-coupled cAMP receptor, an adenylyl cyclase, and phosphodiesterases, self-organized cAMP signals are initiated within aggregation centers and relayed by surrounding cells, resulting in outwardly propagating waves that guide the collective migra-

tion of up to 100,000 cells during the early stages of the developmental program. In addition to their role as chemoattractant, the periodic cAMP signals trigger profound changes in the transcriptome (6, 7). It has been known since the 1970s that optimal development is promoted by repeated stimuli at the “correct” frequency and suppressed by continuous stimulation (8, 9), yet the underlying mechanism for this pattern of response has been elusive. We report here that developmental progression requires a conserved transcription factor, GtaC, which undergoes oscillatory nucleocytoplasmic shuttling. The unique mode of regulation of GtaC by extracellular cAMP can explain why correctly timed repeated stimuli are required for proper gene expression and development.

Results

cAMP Waves Drive Oscillatory Nucleocytoplasmic Shuttling of GtaC

In a forward genetic screen using restriction endonuclease-mediated integration (10), we isolated a mutant that failed to aggregate when plated clonally on bacteria lawns (fig. S1A). The insertion occurred in the second exon of the *gtaC* gene, which encodes a GATA family zinc finger transcription factor (fig. S1B), and *gtaC*[−] cells, generated via homologous recombination, recapitulated the phenotype of the original mutant (fig. S1, A and B). When plated on non-nutrient agar, which synchronizes the initiation of development, the aggregation of *gtaC*[−] cells was delayed and impaired compared to that of wild-type cells (fig. S1C). The application of extracellular pulses of cAMP, which effectively restores development in several aggregationless mutants identified previously (11, 12), failed to rescue development in *gtaC*[−] cells (fig. S1D). The defect was rescued by the expression of green fluorescent protein (GFP)-tagged

GtaC, but not GFP-GtaC^{C-S}, in which the four conserved cysteines in the zinc finger domain were mutated to serines (fig. S1, A and C).

An intriguing observation was made when we examined the localization of GFP-GtaC. It underwent oscillatory nucleocytoplasmic shuttling during early development (Fig. 1A and movie S1). This behavior was most apparent 2.5 to 4.5 hours after starvation, a stage when propagating cAMP waves occur (13). On the contrary, little GFP-GtaC was found in the nucleus of growing cells or cells aggregated into moundlike structures later during development (movie S2). The period of shuttling, 6.8 ± 0.6 min, was similar to that reported for spontaneous cAMP oscillations (Fig. 1C). Further, whereas shuttling appeared synchronized among the cells within a narrow microscopic field (Fig. 1, A and B; fig. S2, A and B; and movie S1), at lower magnification, it became clear that the nuclear localization of GFP-GtaC propagated across the field as a wave with a speed ($\sim 100 \mu\text{m}/\text{min}$) similar to that of cAMP waves (fig. S3 and movie S3) (13, 14). The fact that the rising phase of an approaching cAMP wave causes a transient increase in cell polarity and rate of motility (14, 15) allowed us to align the localization of GtaC with cAMP changes. We observed that the cells became slightly elongated and the speed of movement increased three- to fourfold when GFP-GtaC localized to the cytoplasm, and they were rounder and less motile when GFP-GtaC was in the nucleus (Fig. 1, A and C; fig. S2, A and B; and movie S1). This implies that GtaC shifts to the cytoplasm during the rising phase of the cAMP wave and reenters the nucleus during the falling phase.

To directly assay the effect of cAMP, we monitored the behavior of GFP-GtaC in isolated cells during application and removal of stimuli. When exposed to persistent and uniform cAMP stimulation, after a brief lag GtaC shifted from the nucleus to the cytoplasm with a half-life of ~ 65 s (Fig. 1D and movie S4) and remained in the cytoplasm for as long as the stimulus was present (more than 30 min). When the stimulus was removed, GtaC reaccumulated in the nucleus with a half-life of ~ 95 s (Fig. 1D and movie S5). The nucleus-to-cytoplasm translocation depended on cAMP receptor occupancy because it did not occur in cells lacking the receptors cAR1 and cAR3 (Fig. 1D and movie S6). In addition, robust shuttling could also be observed in cell suspensions when pulses of cAMP were applied at 6-min intervals (fig. S2C). Under this condition, each cAMP addition triggered an amplified response, resulting in elevated cAMP levels for about 1 to 2 min, which then declined to basal levels right before the next pulse (16). Consequently, in each stimulation cycle, the percentage of cells with nuclear GtaC decreased first, reached a minimum at 3 min, and then returned to the initial level at the end (fig. S2C). Together, these results indicate that nucleocytoplasmic shuttling of GtaC is driven by periodic occupancy of the surface receptor from self-organized cAMP oscillations.

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GtaC Shuttling Depends on Its Nuclear Localization Signal and Cyclic Phosphorylation

We constructed a series of mutants to examine the involvement of different regions of GtaC in its dynamic behavior. Because many oscillatory transcription factors reported previously are involved in negative feedback loops where their level or activity is down-regulated by target gene products (17–20), we first tested whether shuttling requires the zinc finger DNA binding domain. GFP-GtaC^{C-S} showed no dominant effect when expressed in the wild-type background (fig. S4A), and the kinetics and extent of its nucleocytoplasmic relocation during persistent or repetitive stimulation were indistinguishable from that of the intact protein (Fig. 1D, fig. S2C, and movie S7), indicating that the zinc finger domain is dispensable. In contrast, when a nearby region con-

taining a putative nuclear localization signal (NLS) was deleted (GtaC^{ΔNLS}) or mutated (GtaC^{KR-A}), GtaC could no longer localize to the nucleus or rescue the aggregation defect of *gtaC*[−] cells (Fig. 2, A and B, and fig. S4, A and B). Removing most of the C terminus following the zinc finger domain (GtaC^{Δ543–587}) or the N-terminal 180 residues did not significantly impair the localization or function of GtaC (Fig. 2, A and B, and fig. S4, A and B). However, truncations from the N terminus (GtaC^{Δ1–228}, GtaC^{Δ1–280}, and GtaC^{Δ1–345}) led to a complete loss of regulation and constitutive nuclear localization that still depended on the NLS activity (Fig. 2, A and B, and fig. S4B). Because the N-terminal region itself did not have apparent nuclear export activity or consensus sequence (fig. S4C), we speculate that the N terminus of GtaC (involving especially residues 180 to

228) may facilitate cAMP-induced relocation by neutralizing the action of the endogenous NLS.

We noticed that the nuclear-to-cytoplasmic translocation of GtaC coincided with a shift in its electrophoretic mobility, which required cAMP receptor occupancy (Fig. 2C and fig. S5A). The slower migrating species could be recognized by antibodies to phospho-serine and phospho-threonine, and the shift was reversed by treatment with λ-phosphatase, indicating that it was caused by cAMP-induced phosphorylation (Fig. 2D). Furthermore, periodic changes in the phosphorylation status of GtaC were detected in response to oscillatory cAMP stimulation: GtaC became phosphorylated and then dephosphorylated when levels of cAMP increased and decreased sequentially during each cycle (Fig. 2E). These observations indicate that localization of GtaC is closely linked

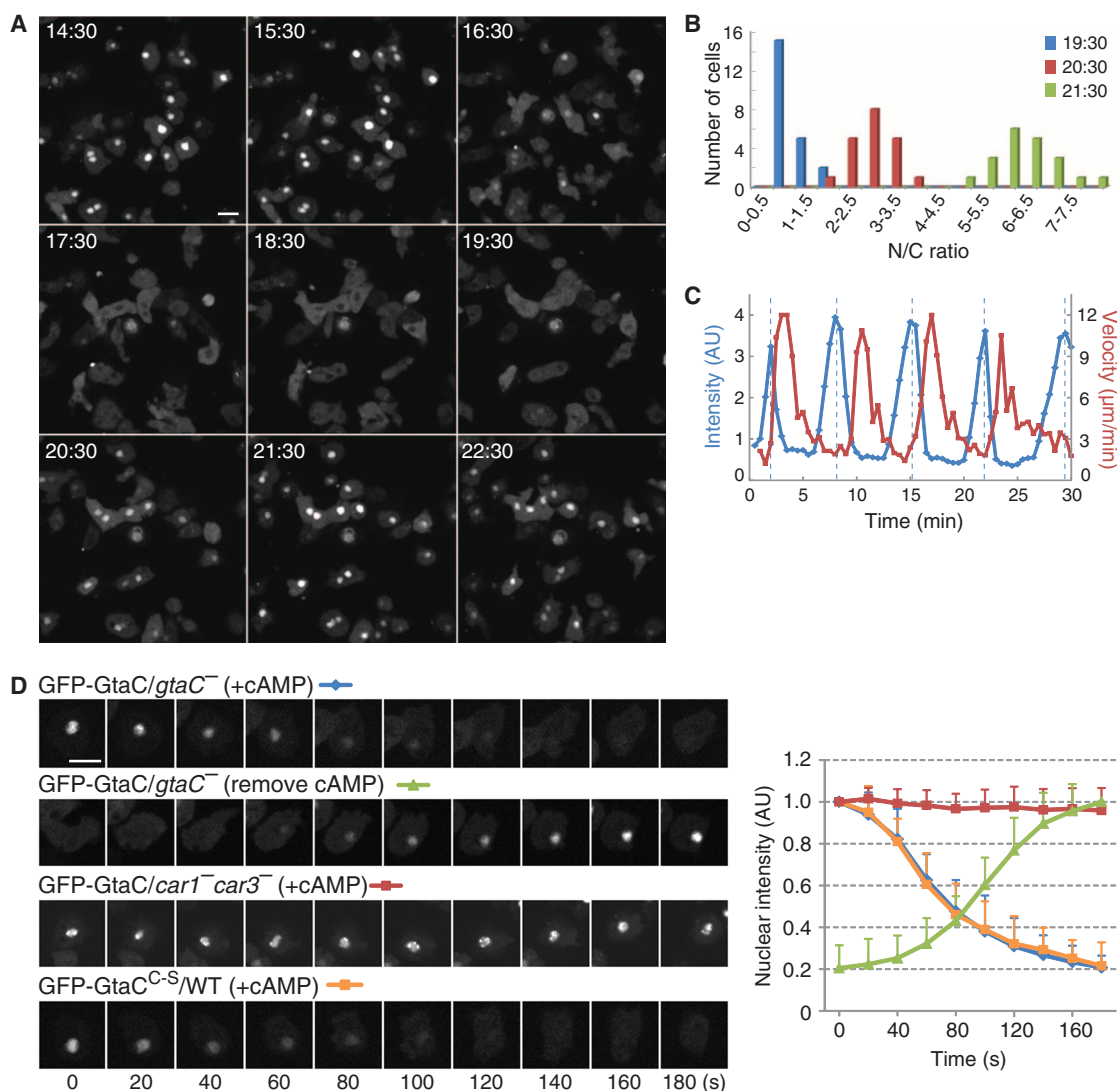


Fig. 1. cAMP oscillations drive the nucleocytoplasmic shuttling of GtaC during early development. (A) Time-lapse microscopic images (from movie S1) showing oscillatory nuclear enrichment of GFP-GtaC in a monolayer of cells. Time, minutes:seconds. (B) Histogram of the distribution of nucleus/cytoplasm fluorescence intensity ratio (N/C) at the indicated time points. (C) Plot of fluorescence intensity of GFP-GtaC (intensity peaks indicated by dashed lines) and speed of cell movement over a 30-min period. (D) Image sequence showing

nuclear-to-cytoplasmic translocation of GFP-GtaC upon uniform cAMP stimulation (first panel, $n = 42$). The translocation was reversed when a stimulus applied for about 10 min was removed (second panel, $n = 34$), was blocked in cells lacking the cAMP receptors (third panel, $n = 45$), but was not affected by the zinc finger domain mutations (fourth panel, $n = 40$). Quantification of the changes in nuclear fluorescence intensity is shown on the right. Data are means + SD. AU, arbitrary units; WT, wild type. Scale bar, 10 μm.

to its phosphorylation states. Purified phosphorylated and dephosphorylated GtaC displayed different patterns of degradation during limited trypsin digestion, implying a potential conformational change upon phosphorylation (fig. S5B).

To investigate the functional significance of phosphorylation, we sought to map the relevant sites. Two consensus protein kinase A (PKA; effector of intracellular cAMP) recognition sites (S^{472} and T^{473}) were found within the NLS. Alanine substitutions of the two sites, however, did not affect phospho-cycling (fig. S5C). Phosphoproteomic analyses revealed a cluster of three serine/threonine residues (T^{376} , S^{379} , and S^{380}) that were phosphorylated in a cAMP-dependent manner (Fig. 2F). We expressed a construct con-

taining alanine substitutions at these positions as well as mutations in the zinc finger domain ($GtaC^{3A+C-S}$) in the wild-type background. With persistent or repetitive cAMP stimulation, the mutant protein still displayed decreased electrophoretic mobility, but the apparent molecular weight shift was significantly smaller than that displayed by the wild-type protein (Fig. 2, G and H). Furthermore, cAMP-driven nucleocytoplasmic shuttling was impaired (Fig. 2I), confirming the role of the S/T cluster in regulating GtaC localization.

The three mapped phospho-residues are predicted to be recognition sites of two classes of kinases: glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase (MAPK). Deleting the two *Dictyostelium* MAPKs, ErkA and ErkB,

had little effect on GtaC phosphorylation (fig. S5D). In contrast, in cells lacking GskA, the homolog of GSK3, the stimulus-induced mobility shift was greatly reduced relative to that observed in the wild-type cells (fig. S5E). Furthermore, by adding purified Flag-GskA in vitro, the full extent of shift could be restored (fig. S5F). Together, these results suggest that the cAMP-induced phosphorylations likely induce conformational changes that coordinate with the N terminus of the protein to counteract the activity of the NLS and promote cytoplasmic localization. Because translocation was not abolished by $GtaC^{3A}$ mutation or disruption of GskA (Fig. 2I and fig. S5G), it may also involve the yet unidentified phosphorylation(s) that produces the residual mobility shift or other regulations.

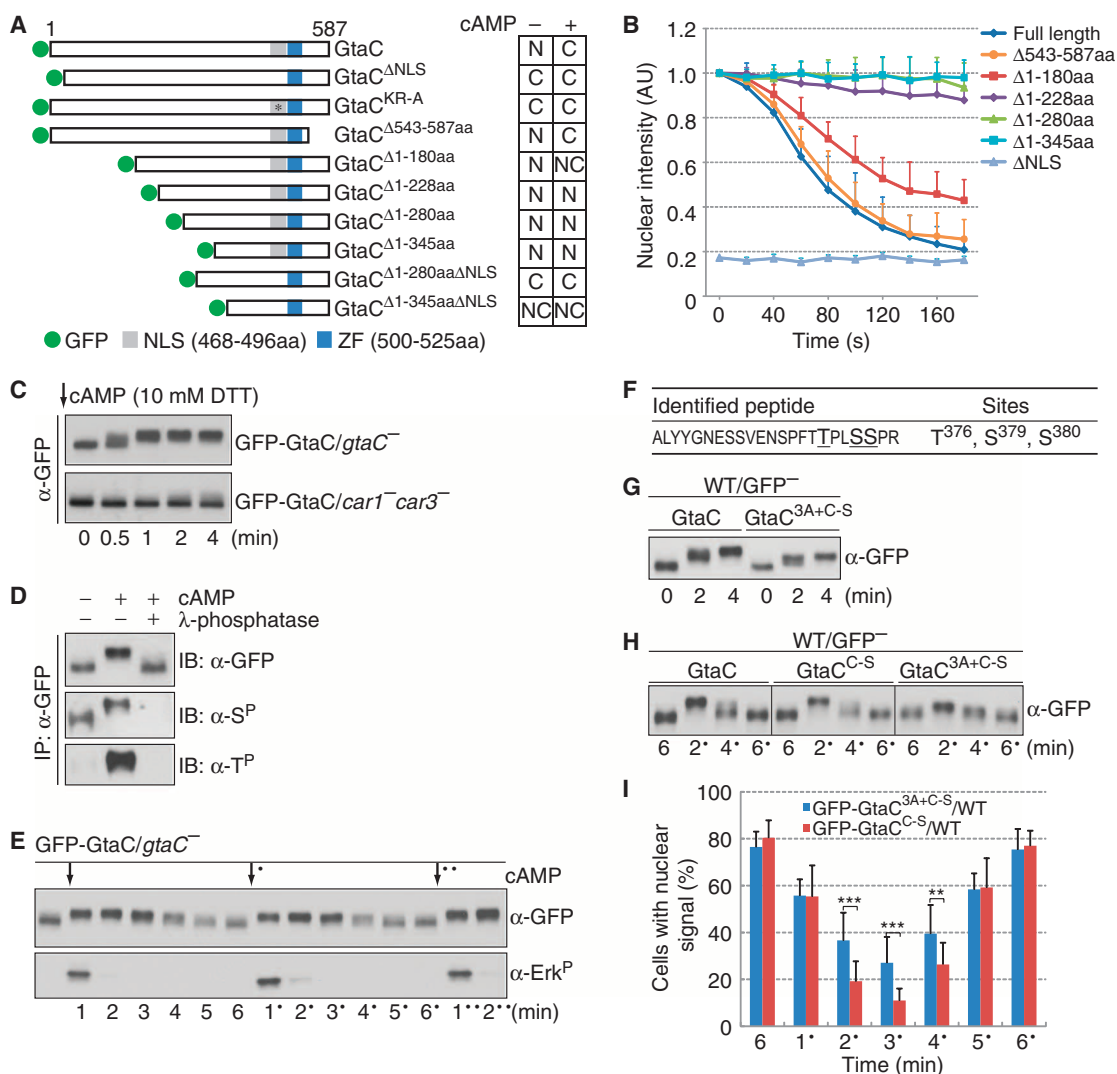


Fig. 2. Regulation of GtaC shuttling. (A) (Left) Schematics of GFP-GtaC and the mutant forms used for localization study. ZF, zinc finger DNA binding domain. Right panel summarizes the localization of WT and mutant proteins before and 5 min after uniform cAMP stimulation (C, predominantly cytoplasmic; N, predominantly nuclear; NC, in both compartments). (B) Quantification of the changes in nuclear fluorescence intensity after uniform cAMP stimulation. Data are means $\pm$ SD ($n \geq 40$). (C) Western blot analysis of samples collected after persistent cAMP stimulation [cAMP degradation blocked by dithiothreitol (DTT)]. (D) The cAMP-stimulated mobility shift was reversed by treatment with λ -phosphatase. The slower migrating species was recognized by anti-

bodies to phospho-serine and phospho-threonine. (E) Pulses of cAMP applied at 6-min intervals (indicated by the arrows) induced periodic changes in the apparent molecular weight of GFP-GtaC and the phosphorylation status of the ErkB kinase (dots by arrows and numbers indicate the pulsing cycle numbers). (F) Phosphoproteomic analysis identified three cAMP-dependent phosphorylation sites (underlined). (G and H) GFP-GtaC^{3A+C-S} displayed reduced mobility shift in response to persistent (G) or pulsatile (H) cAMP stimulation. (I) Shuttling of GFP-GtaC^{C-S} and GFP-GtaC^{3A+C-S} in response to pulsatile stimuli. Graph quantitates the percentage of cells with discernible nuclear signal of GtaC. Data are means $\pm$ SD ($n \geq 150$). ** $P < 0.01$; *** $P < 0.001$.

GtaC Shuttling Regulates Development and Gene Expression

To examine the effects of disrupted shuttling, we added an exogenous NLS, which kept GtaC predominantly in the nucleus (fig. S6A and movie S8). The NLS^{ex}-GFP-GtaC-expressing cells entered development prematurely. When assayed for migration in a defined gradient of cAMP, 2- to 3-hour (post-starvation) NLS^{ex}-GFP-GtaC cells displayed motility and directionality parameters comparable to 4- to 5-hour GFP-GtaC cells (Fig. 3A and movies S9 to S11). Consistently, on non-nutrient agar, NLS^{ex}-GFP-GtaC cells assembled into moundlike structures 2 to 3 hours earlier than wild-type or GFP-GtaC cells (Fig. 3B and fig. S6B). However, the size of these structures, which reflects the number of cells synchronized to aggregate, was greatly reduced and development often arrested at the mound stage (Fig. 3B and fig. S6B).

Rapid and aberrant development was also observed with cells expressing the mutant forms of GtaC (GtaC^{3A}, GtaC^{Δ1-228}, GtaC^{Δ1-280}, and GtaC^{Δ1-345}) that were defective in cAMP-induced translocation (fig. S6C). Moreover, a similar phenotype was reported for *gskA*⁻ (21), consistent with its demonstrated role in regulating GtaC phosphorylation and translocation (fig. S5, E to G).

To uncover gene expression changes associated with differential development, we performed RNA sequencing (RNA-Seq) analyses with wild-type, *gtaC*⁻, GFP-GtaC/*gtaC*⁻, and NLS^{ex}-GFP-GtaC/*gtaC*⁻ strains (Fig. 3C and fig. S7A). We calculated the maximal correlation between the transcriptional profile of each individual strain and that of the wild type at each development time point using genes whose transcripts exhibited changes in at least one strain (22). The initial profiles of all four strains were similar (0 hour in Fig.

3C). The profile of *gtaC*⁻ was similar to that of the wild type up to 1 hour of development but arrested thereafter (Fig. 3C), consistent with GtaC being essential for developmental progression (fig. S1). GFP-GtaC/*gtaC*⁻ resembled the wild type throughout the time course, suggesting that GFP-GtaC behaved like the endogenous protein and complemented the *gtaC*⁻ mutant (Fig. 3C). In contrast, NLS^{ex}-GFP-GtaC markedly accelerated the profile at early time points although it was expressed about two- to threefold lower than GFP-GtaC at the protein level (Fig. 3C). The overall gene expression trends correspond well to the morphological phenotypes of the respective strains (Fig. 3B and fig. S1C).

We then examined developmentally induced transcripts in more detail. Compared to *gtaC*⁻, 1008 transcripts exhibited significantly higher abundance at 5 hours in both wild-type and GFP-GtaC/*gtaC*⁻

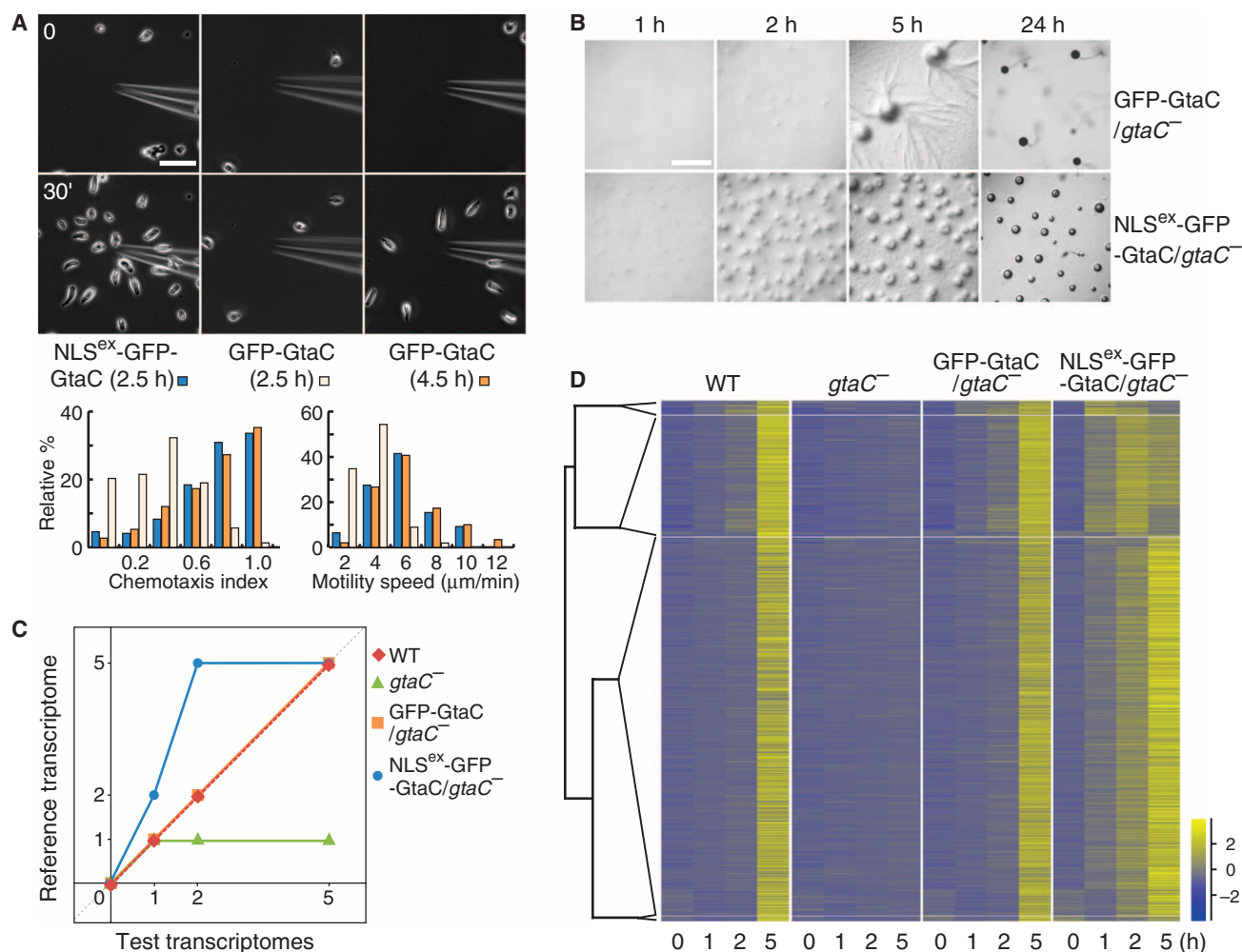


Fig. 3. GtaC shuttling is important for proper gene expression and development progression. (A) Cells developed on non-nutrient agar for 2.5 or 4.5 hours were exposed to cAMP gradients generated by a micropipette. (Top) Snapshots taken at the indicated time points. (Bottom) Quantification of chemotaxis index and motility speed ($n \geq 175$). Scale bar, 50 μ m. (B) Cells expressing NLS^{ex}-GFP-GtaC developed precociously on non-nutrient agar. Scale bar, 2 mm. (C) Maximal similarity plot between the four test transcriptomes (WT, *gtaC*⁻, GFP-GtaC/*gtaC*⁻, and NLS^{ex}-GFP-GtaC/*gtaC*⁻) at each development time point (x axis) and the WT reference transcriptome (y axis) generated from

10,280 genes whose transcripts exhibited change in at least one strain. The dashed gray line represents a hypothetical comparison between perfectly matching developmental profiles. (D) The heat maps generated from RNA-Seq analyses represent the patterns of change in standardized mRNA abundance of 1008 selected transcripts (rows) over four time points (columns) in the indicated strains. These transcripts were significantly more abundant in WT and GFP-GtaC/*gtaC*⁻ cells at 5 hours than in *gtaC*⁻ cells. They were grouped by hierarchical clustering analysis on the NLS^{ex}-GFP-GtaC/*gtaC*⁻ data over four time points. Colors represent relative mRNA abundances (see scale).

gtaC[−] cells, and most of these transcripts (796) were precociously expressed in NLS^{ex}-GFP-GtaC/*gtaC*[−] cells (Fig. 3D). This trend was also found at the 1- and 2-hour time points (fig. S7B). A subset of GtaC-dependent genes (18% in the 1- and 2-hour list and 8% in the 5-hour list) was significantly underexpressed in NLS^{ex}-GFP-GtaC/*gtaC*[−] compared to GFP-GtaC/*gtaC*[−] cells. Gene Ontology (GO) analyses revealed an enrichment of GO terms associated with GtaC-dependent genes, including “cAMP biosynthetic process” and “cell-cell recognition,” two essential processes during early development (fig. S7, C to E). Together, these experiments demonstrate that GtaC shuttling is required for proper control of developmental gene expression and development progression.

Nuclear GtaC Is Necessary But Not Sufficient for Development

The persistent nuclear localization rendered by NLS^{ex} enabled further investigation of the requirement of GtaC and cAMP during development. When wild-type or GFP-GtaC/wild-type cells were repeatedly stimulated at 6-min intervals or allowed to oscillate spontaneously, they became polarized and turned on the expression of two GtaC-dependent genes, *csaA* and *cAR1*, which have been used extensively as markers for development (Fig. 4A and figs. S7, D and E, and S8, A and B). In contrast, continuous stimulation by

sp-cAMPS (a nondegradable analog of cAMP) suppressed these events (Fig. 4A and fig. S8, A and B). The suppression by sp-cAMPS could be bypassed by expressing NLS^{ex}-GFP-GtaC (Fig. 4A and fig. S8, A and B). Thus, in wild-type cells, pulses of cAMP are more effective as signals in promoting development than continuous stimuli because the former allow repeated nuclear entry of GtaC, whereas the latter keep GtaC in the cytosol.

To test whether nuclear localization of GtaC alone is sufficient to drive development, we used cells lacking the major adenylyl cyclase (*AcaA*) that is necessary for cAMP production. In *acaA*[−] cells, which do not have spontaneous oscillations, development and the expression of *csaA* and *cAR1* required repeated cAMP stimuli (Fig. 4B and fig. S8C). NLS^{ex}-GFP-GtaC by itself was not able to circumvent this requirement—gene expression and developmental changes did not occur in these cells unless external cAMP stimuli were applied (Fig. 4B and fig. S8C). These experiments indicate that nuclear GtaC is necessary but not sufficient to fully relay cAMP signals and that gene expression needs an additional input from cAMP.

Thus, cAMP signaling appears to play two distinct roles in early development—it acts as a positive regulator of cell differentiation and gene expression but also drives GtaC out of the nucleus and thereby negatively regulates these events. The two seemingly opposing effects encoded in cAMP signaling

may allow cells to respond specifically to oscillatory stimuli, which provide a repeated activation signal and repeated nuclear accumulation of GtaC. We carried out experiments to further explore how the dual effects of cAMP allow cells to interpret dynamic signals.

Cells Process Oscillatory cAMP Signaling to Regulate Development

The fact that gene expression responds to repeated stimuli and that GtaC shuttles in and out of the nucleus suggests that transcription may occur in distinct “units.” To test this possibility, we used the MS2 technique (fig. S9A) (23, 24), which detects nascent messenger RNA (mRNA) at the site of transcription as a fluorescent spot, to directly visualize transcriptional dynamics of the GtaC-dependent target gene, *csaA*. Remarkably, *csaA* transcription spots displayed an oscillatory pattern like the cAMP oscillations and GtaC shuttling at the population level (Fig. 5A). They emerged with a period (5.6 ± 0.8 min) similar to that of the nucleocytoplasmic transit of GtaC (5.5 ± 0.1 min) under these conditions (Fig. 5). The peaks of mRNA production lagged behind those of GtaC nuclear accumulation by about 3 to 3.5 min (Fig. 5). A delay is expected with this approach because of transcriptional initiation, elongation, and accumulation of transcripts that are necessary for observing the transcription “spot” (24). In contrast, cyclic transcription was not observed for *csaA* in the absence of GtaC shuttling or for the housekeeping gene *act5* even in the presence of robust GtaC shuttling (fig. S9, B to D). Therefore, via GtaC shuttling, the dynamics of external signals are transmitted internally and propagated to gene activation.

Next, we examined the impact of varying cAMP signaling inputs on the state and localization of GtaC. GtaC shuttling and its phosphorylation appeared to be synchronized to the rhythm of naturally occurring cAMP oscillations (Figs. 2E and 6A). When lower-frequency input was applied, GtaC responded once to each pulse (Fig. 6A). Conversely, with higher-frequency input, GtaC did not have sufficient time to be dephosphorylated and reenter the nucleus between pulses (Fig. 6A). This observation indicates that GtaC shuttling enables cells to track low-frequency, but filter out high-frequency, signals.

The ability to read and convert periodic cAMP signals into repeated gene activation predicts that the accumulation of GtaC-dependent gene products should increase with the number of consecutive cAMP pulses encountered by the cell rather than with developmental age. This was in fact the case for the expression of both *csaA* and *cAR1* (Fig. 6B and fig. S10A). Moreover, pulses at 6-min intervals, which mimicked the natural oscillation, led to higher levels of both gene products compared to less-frequent pulses (pulses at 12- and 20-min intervals), even when the cAMP concentration in each pulse was adjusted such that an equivalent total amount of stimuli was applied under different conditions (Fig. 6, C and D, and

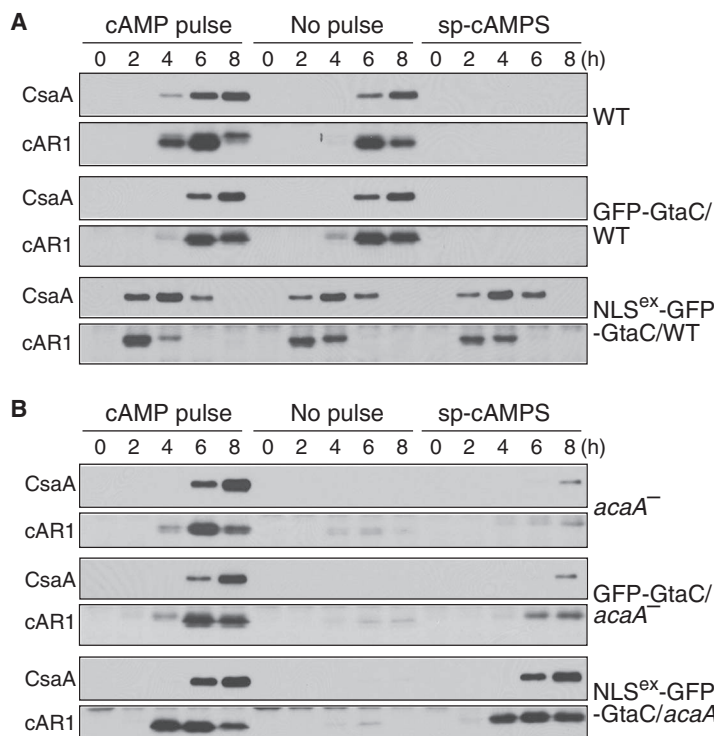


Fig. 4. The dual effects of cAMP during development. Cells were developed under different conditions, and samples were collected at the indicated time points and processed for Western blot analysis. (A) In WT and GFP-GtaC/WT cells, *CsaA* and *cAR1* were induced by repeated cAMP stimuli (cAMP pulse), which reinforce natural oscillations (no pulse), but were suppressed by continuous stimulation (sp-cAMPS). Expressing NLS^{ex}-GFP-GtaC bypassed the inhibitory effect of sp-cAMPS. (B) In *acaA*[−] and GFP-GtaC/*acaA*[−] cells, the expression of *CsaA* and *cAR1* required cAMP pulses (cAMP pulse). With NLS^{ex}-GFP-GtaC, either pulsatile (cAMP pulse) or continuous stimulation (sp-cAMPS) was effective in promoting gene expression, but NLS^{ex}-GFP-GtaC alone (no pulse) was not sufficient.

fig. S10, B and C). In contrast, stimuli that were too frequent to permit GtaC shuttling (Fig. 6A) were filtered out, as predicted, and resulted in little gene expression (Fig. 6C and fig. S10B). These experiments imply the existence of a “counting” mechanism involving GtaC shuttling that ensures gene expression to be properly tuned to the dynamics of cAMP input.

The dual effects of cAMP—it is required for gene expression but drives the key transcription factor, GtaC, out of the nucleus—suggest possible models for the counting mechanism. For example, the activation signal from cAMP could act in concert with GtaC before its exit from the nucleus (Fig. 6E). In this scenario, for a brief time during the rising phase of stimulation, gene expression would be activated and repeated stimuli would lead to reoccurring gene activations. This design resembles the positive edge trigger logic circuit that is frequently used as counter (Fig. 6E). Other similar models may also work. For example, the positive effect of cAMP could set in after GtaC leaves the nucleus and persist for a brief time after GtaC returns to the nucleus. To test the feasibility of using the positive edge trigger circuit to model GtaC-mediated gene expression responses, we carried out computer simulations. We modeled GtaC to be in one of three states: cytosolic (C), nuclear inactive (N), and nuclear active (N*). For simplicity, we assumed that the positive effect of cAMP acted directly on GtaC. Thus, the activation of GtaC (N→N*) and the inhibition of its nuclear localization ($\perp$ C→N) were both regulated by G protein-coupled receptor (GPCR)-mediated processes. The increment of gene products during each stimulation cycle and general turnover rates were incorporated to account for the total level of expression under a given stimulus routine (detailed description is included in the supplementary materials). The simulations successfully recapitulated experimental observations. Transcriptional activation and the accumulation of gene product displayed the same frequency selectivity, with optimal expression occurring with correctly timed stimuli (Fig. 6, F and G).

Discussion

The results presented here reveal a unique mechanism by which oscillatory external signals are used to guide gene expression (Fig. 6H). Surface receptors including GPCRs have been shown to regulate transcriptional response. However, differing from most known examples, in the system we describe here, receptor occupancy leads to the nuclear exit, instead of entry, of the transcription factor. We show that this design can allow cAMP-driven, GtaC-mediated gene expression to occur in a distinct unit in response to each cycle of the repeated stimuli. Consequently, cells are able to count the number of effective stimuli they experience and respond with an appropriate level of gene expression.

Our findings provide several insights critical to the understanding of *Dictyostelium* biology. First, because cAMP waves organize cell aggregation, having gene expression dependent on the number rather than on the absolute concentration

of cAMP stimuli coordinates differentiation among a large population of cells over an expanded territory. Second, in early development, cAMP oscillations have a period similar to that of GtaC phosphocycles. This alignment may be evolutionarily selected because a phospho-cycle deviating from the natural frequency of cAMP oscillations compromises gene expression. Alternatively, the two systems may share regulatory components that keep them synchronized. Third, it is known that the frequency of cAMP oscillations progressively increases as development enters the mound stage (25), which corresponds nicely to a fall in the number of cells with nuclear enrichment of GtaC (movie S2). Thus, the “low-pass filter” property of the system to admit only low-frequency stimuli may allow the gene expression program to properly transition into the next developmental stage. Future studies are needed to reveal in finer detail the molecular mechanism underlying the dynamic regulation of GtaC and to understand how GtaC shuttling influences the expression of individual genes.

Oscillations at varying temporal and spatial scales are being increasingly observed in biological systems. These rhythmic phenomena regulate many important aspects of cell physiology. Adding to previous reports on oscillatory actions in other systems (18, 19, 26–31), pulsing may represent a

general mechanism for gene expression regulation. It will be interesting to find out whether mechanisms similar to the one we describe here operate in other circumstances where gene expression needs to be synchronized spatiotemporally among cell population or linked to repeated experience.

Materials and Methods

Cell Growth and Differentiation

Wild-type and gene deletion cell lines were cultured axenically in HL5 supplemented with antibiotics (32). Cells carrying expression constructs were maintained in HL5 containing G418 (10 to 20 μ g/ml) or hygromycin (50 μ g/ml). Developmental assays were performed as described previously with exponentially growing cells (32). Briefly, for development on bacterial lawn, cells were diluted in HL5 without antibiotics and plated with a suspension of *Klebsiella aerogenes* on SM plate. For development on non-nutrient agar, cells were washed with developmental buffer (DB) and plated on solidified DB agar at a density of 5.2×10^5 cells/cm². For most experiments, development in suspension was carried out by pulsing cells resuspended in DB at 2×10^7 cells/ml with 50 to 100 nM cAMP every 6 min starting from 1 hour. For the experiments presented in Fig. 6 (A to D)

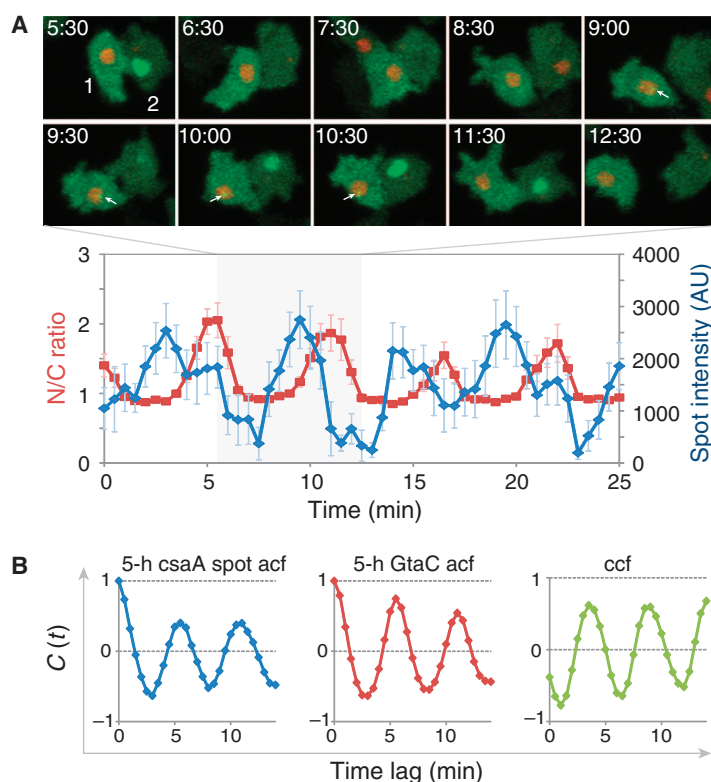


Fig. 5. GtaC shuttling promotes cyclic transcription. (A) Two populations of cells were mixed together to observe GtaC localization and transcription dynamics simultaneously. (Top) Image sequence (maximum intensity projections of three-dimensional stacks) showing GtaC translocation and *csA* transcription in 5-hour (poststarvation) cells at the indicated time points. Cell 1, WT cell, in which an array of MS2 loops was integrated at the 5' end of the *csA* gene, stably expressing MS2-GFP and mRFP-H2B; cell 2, WT cell expressing GFP-GtaC. Transcription was visualized as a green spot (arrows) in the red nucleus. (Bottom) Quantification of the nucleus/cytoplasm fluorescence intensity ratio (N/C) of GFP-GtaC ($n = 10$) and the intensity of the *csA* transcription spot ($n = 12$). Data are means $\pm$ SEM. (B) Autocorrelation (acf) and cross-correlation (ccf) plots.

and fig. S10 (A to C), *crac*⁻ or GFP-GtaC/*crac*⁻ cells were pulsed with different concentrations of cAMP or at different intervals starting from 2 hours. For development in the presence of sp-cAMPS, 1 μ M was included in DB or DB agar.

Gene Disruption

The *gtaC*⁻ cell line was generated from the Ax2 background. The disruption construct consists of a blasticidin S resistance (BSR) cassette from

pLPBLP (33) flanked by two genomic DNA fragments amplified by polymerase chain reaction (PCR) using primers KOF1-KOR1 and KOF2-KOR2, respectively (see table S2 for primer sequences). Through homologous recombination, the region of *GtaC* between +541 and +1323, in which +1 is the first and +2007 is the last nucleotide of the open reading frame, was replaced by the BSR cassette. Knockouts were screened by PCR and confirmed by Southern blotting.

Plasmid Construction

To generate constructs in which GtaC was tagged with GFP or Flag at the N terminus, full-length GtaC was amplified by PCR from genomic DNA using P1 and P2 primers, the two introns were subsequently deleted, and the resulting fragment was cloned into the Bgl II and Spe I sites in pDM317 or pDM320 (34). For deletion studies, N- and C-terminal fragments of GtaC were amplified by PCR and cloned into the Bgl II and Spe I

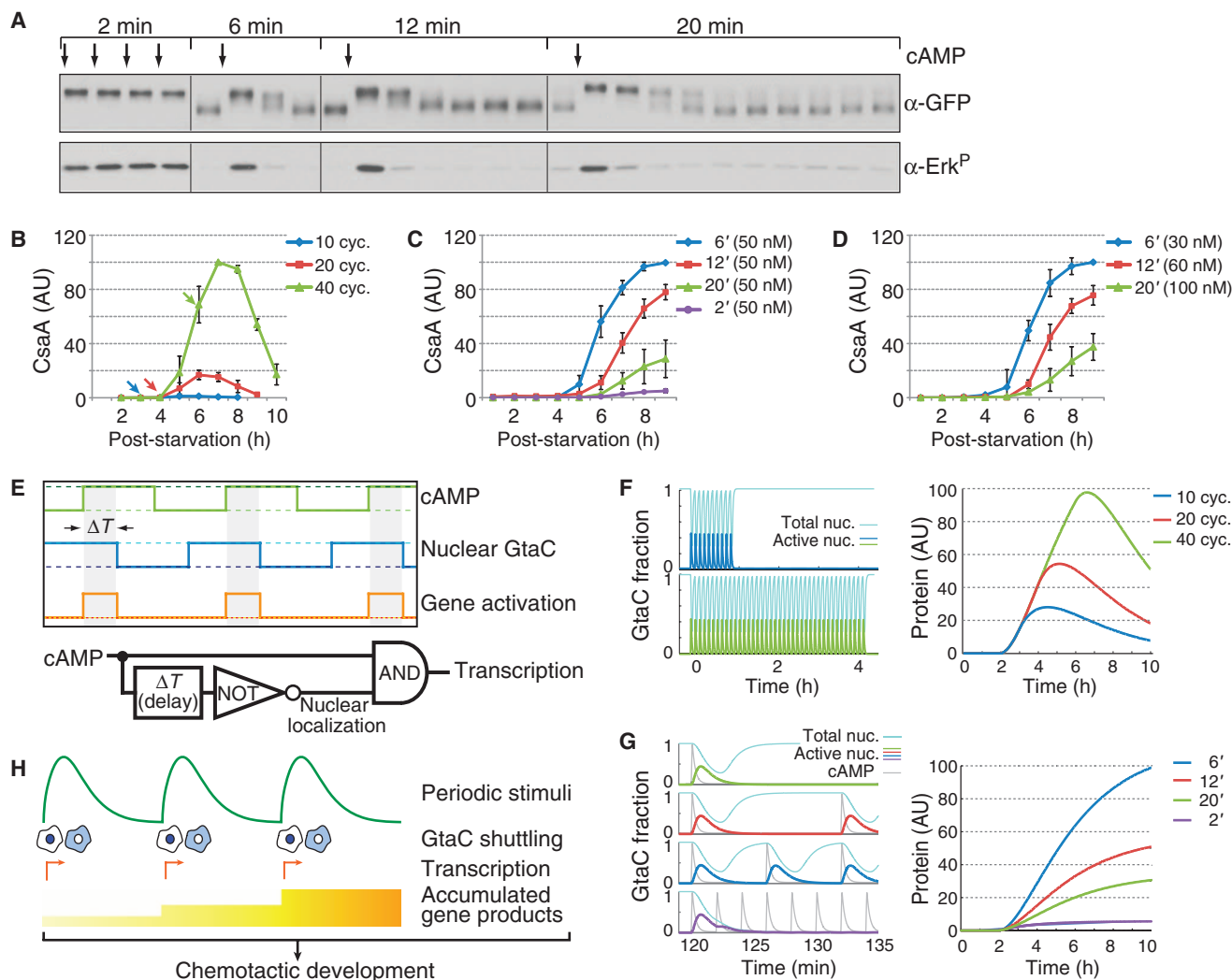


Fig. 6. GtaC shuttling constitutes a cellular mechanism that processes oscillatory cAMP signaling. (A) GFP-GtaC/*crac*⁻ cells were developed with cAMP pulses applied at different intervals. Samples were collected every 2 min. (B) Expression of CsaA protein in *crac*⁻ cells in response to different numbers of cAMP pulses applied at 6-min intervals. Arrows indicate the time points when pulses were discontinued. (C) Expression of CsaA in *crac*⁻ cells in response to stimulations applied at different intervals. (D) Similar experiment as in (C) except that the cAMP concentration in each pulse was adjusted to generate an equivalent total amount of stimuli under different conditions. (E) (Top) A proposed model for cAMP-driven GtaC-mediated gene activation. In each cycle of stimulation (green line), the positive effect of cAMP on gene activation precedes the exit of GtaC from the nucleus (blue line), thereby creating a window of time (ΔT , gray shade) when both factors are present to promote gene expression (orange line). Persistent stimulation (dark green dashed line) drives GtaC out of the nucleus (dark blue dashed line), whereas lack of stimulation (light green dashed line) fails to generate an activation signal, and in both cases, gene expression is not activated (red and yellow dashed lines). This design resembles the “positive edge trigger” logical circuit. The biological circuit

consists of an AND gate with cAMP and nuclear GtaC as two inputs to activate transcription and a delayed NOT gate, by which cAMP controls the nuclear-to-cytoplasmic translocation of GtaC. The circuit becomes active when cAMP level goes from low to high. (F) Simulation showing the effects of the number of cAMP pulses. Ten, 20, or 40 pulses were applied, 6 min apart starting at 2 hours. (Left) Fractions of GtaC in the nucleus and active for 10 (top) and 40 (bottom) pulses. (Right) Protein expression as a function of time. The amount of protein is normalized to the total amount produced by a single pulse. (G) Simulation showing the effects of the pulsing period. Pulses spaced 2, 6, 12, and 20 min apart were applied starting at 2 hours. (Left) cAMP and fractions of GtaC in the nucleus and active. The peak concentration of cAMP is $\sim 4 \mu$ M. (Right) Protein expression as a function of time. Note that with 2-min periods, the new pulse was applied before the GtaC reentered the nucleus; hence, the subsequent pulses did not lead to further activity. (H) Schematic of chemotactic development regulation. Repeated stimuli coupled with nucleocytoplasmic shuttling of GtaC generate units of transcription. Integration of sequential transcriptional activities allows cells to reckon the experience of stimulation and tune the level of gene expression.

sites in pDM448 and pDM317, respectively (34). To generate GFP-GtaC^{C-S}, the cysteine residues at positions 500, 503, 522, and 525 were mutated to serines. To generate GFP-GtaC^{ANLS}, the region containing residues 468 to 496 was deleted. To generate GFP-GtaC^{KR-A}, lysines at positions 469, 470, 494, and 496 and arginines at positions 468 and 471 were mutated to alanines. To generate GtaC construct carrying mutations in the consensus PKA recognition sites, S⁴⁷² and T⁴⁷³ were mutated to alanines. To generate GFP-GtaC^{3A}, T³⁷⁶, S³⁷⁹, and S³⁸⁰ were mutated to alanines. To generate NLS^{ex}-GFP-GtaC, DNA fragment amplified by PCR using primers P2 and P3 was cloned into the Bgl II and Spe I sites in pDM304 (34). To generate Flag-GskA, full-length GskA was amplified by PCR from genomic DNA using P4 and P5 primers and cloned into the Bgl II and Spe I sites in pDM320.

cAMP Stimulation and Immunoblotting

For the experiments presented in Fig. 2 (C and G) and fig. S5 (A, D, and E), cells developed in suspension for 2 hours were washed with cold DB, resuspended in DB to a density of 2×10^7 cells/ml, and kept on ice before stimulation. Cells were stimulated at room temperature with the addition of cAMP with a final concentration of 1 μ M (Fig. 2, C and G, and fig. S5, D and E) or ranging from 0.3 to 300 nM (fig. S5A), lysed with 3 $\times$ sample buffer, and boiled for 5 min. For the experiments presented in Fig. 2 (E and H) and fig. S5C, cells developed in suspension for 2.5 to 3 hours were collected right before or at different time points after the addition of one pulse of cAMP, lysed with 3 $\times$ sample buffer, and boiled for 5 min. Combining with mutations in the zinc finger domain allowed us to examine the phosphorylation status of GtaC^{3A} and GtaC^{S472AS473A} in the wild-type background without compromising development or cell responsiveness to cAMP. To best observe the mobility shift of GtaC, proteins were separated on a 7.5% tris-HCl polyacrylamide gel (Criterion; Bio-Rad) and run at a constant voltage of 150 V for 110 min. To check the expression of CsaA and cAR1, cells were collected during development, pelleted, resuspended in DB to a final concentration of 5×10^7 cells/ml, and lysed with 3 $\times$ sample buffer without boiling. Proteins were separated on a 4 to 15% tris-HCl polyacrylamide gel.

Western blotting was carried out as described previously (35). Equal protein loading was verified by staining the membrane with Coomassie Brilliant Blue after immunoblotting. We obtained antibody to GFP (catalog no. 11814460001) from Roche, antibody to phospho-serine (catalog no. 05-1000) from Millipore, and antibody to phospho-threonine (catalog no. 9381) from Cell Signaling Technology. Antibody to phospho-p44/42 MAPK (catalog no. 4370) from Cell Signaling Technology was used to detect phosphorylation of the ErkB kinase. Antibody to CsaA (33-294-17-s) was from the Developmental Studies Hybridoma Bank.

Micropipette Assay

gtaC⁻ cells expressing GFP-GtaC or NLS^{ex}-GFP-GtaC were developed on the surface of non-nutrient

agar. Differentiated cells were diluted in DB, plated on coverslip chambers (Lab-Tek, Nalge-Nunc), allowed to adhere for ~10 to 15 min, covered with DB, and then exposed to a micropipette filled with 10 μ M cAMP. Chemotaxis was recorded by time-lapse video using an inverted microscope (Zeiss Observer.Z1) with a 10 $\times$ /0.3 objective. Images were acquired at 30-s intervals using the AxioVision software. Motility speed and chemotaxis index were measured as described previously (36).

Image Acquisition

For the experiments presented in Fig. 1A and fig. S2A, GFP-GtaC/*gtaC*⁻ cells were plated on a chambered coverslip at a density of 3.5×10^5 cells/cm² and submerged in DB. After 3 to 3.5 hours, time-lapse video was obtained using a Yokogawa CSU10 spinning disc confocal microscope equipped with a 40 $\times$ /0.3 oil objective. Images were acquired at 30-s intervals using the SlideBook software. For the experiments presented in fig. S3, cells were washed with DB and plated on the surface of a thin layer of DB agar in a one-well coverslip chamber at a density of 4.5×10^5 cells/cm². Time-lapse video was taken at 4 hours using a Zeiss Observer.Z1 inverted microscope equipped with a 10 $\times$ /0.3 air objective. Images were acquired at 1-min intervals with GFP illumination.

For the experiments presented in Figs. 1D and 2 (A and B) and figs. S4B, S5G, and S6A, cells developed in suspension for 2 hours were collected, washed, and diluted in DB. Cells (360μ l) at a density of 1.4×10^5 cells/ml were plated in an eight-well coverslip chamber. Cells were allowed to adhere for 10 to 15 min. Forty microliters of 1 μ M cAMP was added for stimulation. For the “remove cAMP” experiment presented in Fig. 1D, cells were first stimulated as described above, and the stimulus was applied for at least 5 min for the nuclear-to-cytoplasmic translocation to complete. Imaging started when the stimulus was replaced with DB. GtaC relocation was captured by time-lapse video using an UltraView spinning disc confocal microscope (DM 16000; PerkinElmer) equipped with a 40 $\times$ /1.25–0.75 oil objective. Images were acquired at 20-s intervals using the SlideBook software.

For the experiments presented in Fig. 2I and fig. S2C, cells were developed in suspension for 2.5 hours. Cells were collected right before or at different time points after the addition of one pulse of cAMP (100 nM) and fixed immediately in phosphate-buffered saline (PBS) containing 3.7% formaldehyde. Fixed cells were washed once with PBS and plated on a chambered coverslip. Images were acquired using the SlideBook software on a Yokogawa CSU10 spinning disc confocal microscope equipped with a 40 $\times$ /0.3 oil objective.

For the experiments presented in fig. S8 (B and C), cells were developed under different conditions and plated in an eight-well coverslip chamber. Images were acquired with phase illumination on a Zeiss Observer.Z1 inverted microscope equipped with a 20 $\times$ /0.3 air objective.

To visualize transcription of *csaA* and *act5*, MS2 knock-in cells were cotransformed with plas-

mids encoding MS2-GFP that binds to nascent mRNA at the transcription site and mRFP-H2B that labels the nucleus (24). To image transcription and GtaC simultaneously, MS2-GFP + mRFP-H2B/wild-type and GFP-GtaC/wild-type cells were washed with KK2 buffer (20 mM potassium phosphate, pH 6.2), and equal numbers of cells were mixed and plated on 2% of KK2 agar at a density of 3.5×10^5 cells/cm². After 7 min for cells to settle, the buffer was removed and the cells were incubated in a humidified chamber. Agar was inverted onto an imaging dish before imaging and covered with mineral oil to prevent desiccation. Cells were captured using an A1R resonant scanning confocal microscope (Nikon) equipped with a 60 $\times$ /1.49 oil objective and a controllable XY stage with a piezo attachment for rapid three-dimensional capture at multiple xy positions. Three-dimensional stacks (35 slices; 350-nm z step) were captured every 30 s for 25 min.

Image Analysis

ImageJ [National Institutes of Health (NIH)] was used for most analyses. To quantitate fluorescence intensity at single-cell level as presented in Figs. 1 (B and D) and 2B and figs. S2B, S5G, and S6A, the background-corrected mean pixel intensity of the nuclear and/or the cytoplasmic region was measured. To generate the histogram presented in Fig. 1B, the nucleus/cytosol ratios from 20 cells were binned to ranges of 0.5 intervals, and the frequency of each ratio range was plotted. For graphs presented in Fig. 2I and fig. S2C, the percentage of cells with greater nucleus/cytosol intensity ratio was determined. For quantification of length/width ratio as presented in fig. S8 (B and C), the distance from the leading edge, as defined by the front of protrusions, to the trailing tail was taken as cell length, and the width of the leading edge, as cell width.

To quantitate the period of nuclear intensity oscillations as presented in Fig. 1C, the response of the cell population as a whole was captured by measuring the average fluorescence intensity over the entire image using a similar method described previously (25). Briefly, the original images from movie S1 were processed with the smooth function in ImageJ. The smoothed images were then subtracted from the original images. The average intensity of all pixels of the resultant images was calculated and plotted against time. Higher intensities correspond to images in which most cells showed nuclear enrichment of GFP-GtaC. Peaks of the intensity plot were interpolated from the zero points of the difference plot, and the intervals between peaks were used to calculate the periodicity. To quantitate speed of movement for Fig. 1C and fig. S2B, images were analyzed using the MTrackJ plug-in (E. Meijering, Erasmus MC, University Medical Center Rotterdam) to generate velocity data. Cells that moved out of the imaging field during the movie were excluded for analysis. In Fig. 1C, the average velocity of 10 cells was plotted against time.

To quantitate fluorescence intensities at the transcriptional sites for Fig. 5 and fig. S9 (B to D), Velocity 3D Image Analysis Software (PerkinElmer)

was used to detect spots and measure intensity values. Only cells that stayed entirely within the imaging field during the movie were analyzed. Background intensities were measured from $5 \times 5 \times 5$ voxel cubes applied to cell bodies. Spot intensities at transcriptional sites were calculated after background subtraction. The temporal autocorrelation function of GtaC and the transcriptional spot are given by the following equation

$$C(t) = \frac{(I(t) - \mu) \cdot (I(0) - \mu)}{\sigma^2}$$

where $I(t)$ is the N/C ratio of GtaC fluorescence intensity or the fluorescence intensity of the transcriptional spot at time t , and μ and σ are the mean and SD, respectively, of each. The temporal cross-correlation function is given by the following equation

$$C_{\text{GtaC-Spot}}(t) = \frac{(I_{\text{GtaC}}(0) - \mu_{\text{GtaC}}) \cdot (I_{\text{Spot}}(t) - \mu_{\text{Spot}})}{\sigma_{\text{GtaC}} \sigma_{\text{Spot}}}$$

where $I_x(t)$ is the N/C ratio of GtaC fluorescence intensity or the intensity of the transcriptional spot at time t , and μ_x and σ_x are the mean and SD of I_x , where $x = \text{GtaC}$ or Spot . Because the period of spontaneous cAMP oscillations often varies, data were not averaged between experiments.

Statistical significance and P values ($P < 0.05$ was considered significant) were determined using the two-tailed Student's t test. Mean values $\pm$ either SD or SEM were reported as indicated in the figure legends. Statistics were derived from three independent experiments for Figs. 1D, 2 (B and I), 3A, and 6 (B to D) and figs. S2C, S5G, S6A, S8 (B and C), and S10.

Immunoprecipitation and Phosphatase Treatment

GFP-GtaC/*gtaC*[−] cells were collected before and after cAMP stimulation, lysed at a density of 2×10^7 cells/ml by adding equal volume of 2× lysis buffer [20 mM sodium phosphate (pH 7.2), 100 mM NaCl, 1% (v/v) NP-40, 100 mM NaF, 50 mM sodium pyrophosphate, 4 mM Na₃VO₄, 2× Complete EDTA-free protease inhibitor mixture (Roche, catalog no. 11873580001)], and incubated on ice for 5 min. Lysates were centrifuged at 16,000g for 5 min to remove debris. Supernatant (600 μl) was incubated with 10 μl of ChromoTek GFP-Trap beads (catalog no. ACT-CM-GFA0050) at 4°C for 2 hours. After washes with buffer [10 mM sodium phosphate (pH 7.2), 50 mM NaCl, 0.5% NP-40, 1× Complete EDTA-free protease inhibitor mixture], the beads were washed with and resuspended in 20 μl of phosphatase buffer (New England Biolabs, catalog no. P0753). Reaction was initiated by the addition of 0.5 μl of λ-protein phosphatase, allowed to proceed for 20 min at 30°C, and stopped by the addition of 15 μl of 3× sample buffer.

GskA Kinase Assay

To purify GtaC as the substrate, GFP-GtaC/*gskA*[−] cells were collected before and after cAMP stimulation, lysed at a density of 1×10^7 cells/ml by

adding equal volume of 2× lysis buffer [50 mM tris (pH 7.5), 200 mM NaCl, 1% NP-40, 100 mM NaF, 50 mM sodium pyrophosphate, 4 mM Na₃VO₄, 2× Complete EDTA-free protease inhibitor mixture], and incubated on ice for 5 min. One milliliter of cleared lysate was incubated with 30 μl of ChromoTek GFP-Trap beads at 4°C for 1.5 hours. Beads were washed with 1× lysis buffer and kept on ice before assay. To purify GskA, Flag-GskA/*gskA*[−] cells were washed with DB and lysed at a density of 2×10^7 cells/ml by adding equal volume of 2× lysis buffer. One milliliter of cleared lysate was incubated with 15 μl of anti-Flag M2 affinity resin (Sigma, catalog no. F2426) at 4°C for 3 hours. Beads were washed with 1× lysis buffer and elution buffer [25 mM tris (pH 7.5), 100 mM NaCl, 0.1% NP-40]. Flag-GskA was eluted by the addition of 100 μl of elution buffer containing 3× Flag peptide (300 ng/μl) (Sigma, catalog no. F4799) at 4°C for 30 min. For kinase reaction, 15 μl of GFP-GtaC-containing beads was washed with elution buffer and mixed with 40 μl of Flag-GskA-containing eluate. Reaction was initiated by the addition of 13× adenosine 5'-triphosphate (ATP) mix (130 mM MgCl₂, 65 mM DTT, 3.9 mM ATP), allowed to proceed for 15 min at room temperature, and stopped by the addition of protein sample buffer.

Limited Proteolysis

To purify phosphorylated and dephosphorylated forms of GtaC, Flag-GtaC/*gtaC*[−] cells were collected before and after cAMP stimulation. Cells (4×10^8) were lysed through two layers of 5-μm filter membranes (Whatman, catalog no. 110613) in the presence of 6× lysis buffer [120 mM tris (pH 8.5), 150 mM NaF, 6 mM Na₃VO₄, 75 mM sodium pyrophosphate, 6× Complete EDTA-free protease inhibitor mixture]. Lysates were centrifuged at 16,000g for 15 min to remove debris. Triton X-100, NaCl, and glycerol were added to the supernatant to a final concentration of 0.5% (v/v), 0.46 M, and 5% (v/v), respectively. The supernatant was then incubated with 90 μl of anti-Flag M2 affinity resin at 4°C for 3 hours. Beads were washed with washing buffer (1× lysis buffer, 0.5% Triton X-100, 0.46 M NaCl, 5% glycerol) and elution buffer [25 mM tris (pH 7.5), 0.4% Triton X-100, 100 mM NaCl]. Flag-GtaC was eluted by the addition of 300 μl of elution buffer containing 3× Flag peptide (300 ng/μl) at 4°C for 1 hour. Limited proteolysis with trypsin (Sigma, catalog no. T1426) was performed at a protein/protease mass ratio of 1000:1. The reaction was carried out at 37°C, and aliquots were removed at 10, 20, 30, 40, and 60 min. The reaction was stopped by the addition of 3× sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and silver staining.

Whole-Transcriptome RNA-Seq and Computational Analysis

Wild-type (Ax2), *gtaC*[−], GFP-GtaC/*gtaC*[−], and NLS^{ex}-GFP-GtaC/*gtaC*[−] cells were developed on DB agar in duplication. RNA samples were collected at 0, 1, 2, and 5 hours and processed as

described previously (37) with the following modifications to perform multiplexed sequencing. At the final step of library preparation, adaptor-ligated complementary DNAs were indexed by PCR using primers that contain a single specific 7-mer barcode. Every eight indexed samples were pooled, and Illumina-based multiplexed sequencing was performed. baySeq package was used to detect differentially expressed genes (38). GtaC-activated target candidates were identified among differentially expressed genes on the basis of two criteria: (i) expression in wild-type and GFP-GtaC/*gtaC*[−] cells at the indicated time point (1, 2, or 5 hours) is at least twofold higher than that in 0-hour wild-type cells; (ii) expression in *gtaC*[−] at the indicated time point (1, 2, or 5 hours) is at least twofold lower than that in wild-type and GFP-GtaC/*gtaC*[−] cells [false discovery rate (FDR) < 0.05 and likelihood > 0.90]. The precociously activated genes were identified as differentially expressed genes that meet the criteria (FDR < 0.05, likelihood > 0.90, and log₂ ratio > 1) in NLS^{ex}-GFP-GtaC/*gtaC*[−] cells compared to GFP-GtaC/*gtaC*[−] cells at time points before 5 hours. topGO package (version 2.14.0) was used to analyze GO term enrichment with the annotation file (November 2012) from dictyBase.

Computer Simulation of GtaC-Mediated Gene Activation

We consider GtaC to be in one of three states: cytosolic (C), nuclear inactive (N), and nuclear active (N^*). The activation of GtaC ($N \rightarrow N^*$) is regulated by a receptor-mediated process (E). Similarly, nuclear localization ($C \rightarrow N$) is inhibited by a second receptor-mediated process (I). Shuttling to the cytoplasm occurs at a constant rate but is limited to the active form of GtaC. Thus, concentrations of the three states are governed by the ordinary differential equations

$$\begin{aligned} \frac{dN}{dt} &= k_1 \frac{C}{k_M + 1} \left(\frac{V_C}{V_N} \right) - k_2 N \cdot E \\ \frac{dN^*}{dt} &= k_2 N \cdot E - k_3 N^* \\ \frac{dC}{dt} &= k_3 N^* \left(\frac{V_N}{V_C} \right) - k_1 \frac{C}{k_M + 1} \end{aligned}$$

Here, V_N and V_C correspond to the volumes of the nucleus and cytoplasm, respectively, and are required to correct for the varying effect that shuttling between the two compartments of different volumes has on concentrations. We assume that the volume of the nucleus is 1/24th that of the cytoplasm. Note that $N + N^* + C \left(\frac{V_C}{V_N} \right) = \text{constant}$, which we can take to be one.

We assume that both E and I are regulated by cAMP receptor occupancy (U) as linear processes

$$\begin{aligned} \frac{dE}{dt} &= k_E(U - E) \\ \frac{dI}{dt} &= k_I(U - I) \end{aligned}$$

In these equations, receptor occupancy is normalized (ranging from 0 to 1), as are both E and I .

In particular, $U = \frac{cAMP/K_D}{1+cAMP/K_D}$, where K_D is in the order of 200 nM (39). The cAMP signal follows the following equation

$$\frac{dcAMP}{dt} = -k_d cAMP + cAMP_{app}(t)$$

where the applied cAMP signal is $1 \mu M = 5 K_D$. The coefficient k_d represents the degradation of extracellular cAMP by phosphodiesterases, which happens with a half-life of about 10 s (40).

Finally, we model transcription and translation using the following differential equations

$$\begin{aligned}\frac{dM}{dt} &= -k_{mRNA}M + N^* \\ \frac{dP}{dt} &= -k_{prot}P + M\end{aligned}$$

The coefficients k_{mRNA} and k_{prot} are chosen so that the half-lives of the mRNA and protein are 1 and 2 hours, respectively.

We selected the unknown parameters by minimizing a function that penalizes three things: the difference between the experimental and simulated concentrations of nuclear signal to both addition and removal of persistent stimuli, the concentration of active nuclear GtaC 200 s after application of a persistent stimulus, and (the inverse of) the peak nuclear GtaC after application of a persistent stimulus. The parameter search was carried out using the Matlab command `fminsearch`. The parameters used in the simulations are given in table S1.

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Supplementary Materials

[www.sciencemag.org/content/343/6177/1249531/suppl/DC1](#)
Figs. S1 to S10
Tables S1 and S2
Movies S1 to S11

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Super-Eddington Mechanical Power of an Accreting Black Hole in M83

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Mass accretion onto black holes releases energy in the form of radiation and outflows. Although the radiative flux cannot substantially exceed the Eddington limit, at which the outgoing radiation pressure impedes the inflow of matter, it remains unclear whether the kinetic energy flux is bounded by this same limit. Here, we present the detection of a radio-optical structure, powered by outflows from a non-nuclear black hole. Its accretion disk properties indicate that this black hole is less than 100 solar masses. The optical-infrared line emission implies an average kinetic power of 3×10^{40} erg second⁻¹, higher than the Eddington luminosity of the black hole. These results demonstrate kinetic power exceeding the Eddington limit over a sustained period, which implies greater ability to influence the evolution of the black hole's environment.

For an accreting black hole (BH) of mass M , the Eddington luminosity [$L_{\text{Edd}} \approx 1.3 \times 10^{38} (M/M_{\odot})$ erg s⁻¹; where $M_{\odot}$ is solar mass] is the maximum photon power achievable for spherically symmetric, radiatively efficient accretion, and the Eddington accretion rate is the

corresponding mass accretion rate. However, BHs can also release energy through mechanical channels (kinetic energy of jets and winds). The balance between radiative and kinetic output depends partly on the mass accretion rate. For accretion rates ~ 0.03 to 0.5 times the Eddington rate, the BH power output is entirely dominated by thermal disk emission without a jet (1–3), whereas for rates approaching or exceeding the Eddington rate, we expect radiatively driven outflows, possibly coexisting with faster, collimated jets (4, 5).

There is circumstantial evidence that non-isotropic photon luminosity can mildly exceed the Eddington limit (3, 6–8), but can BHs with supercritical accretion have powerful jets, and can the mechanical power P_j exceed L_{Edd} for an extended period of time? Empirical evidence of super-Eddington jet power was presented for a sample of radio-loud quasars (9, 10). However, ambiguities in the BH masses and in the conversion from 151-MHz radio flux to jet power

(11–15) make these results uncertain. In the local universe, shock-ionized gas around ultraluminous x-ray sources (ULX bubbles) provides strong evidence of sustained and powerful mechanical output from sources that are thought to have high accretion rates (16–22). Typical ULX bubbles have sizes of ~ 50 to 300 pc and require a mechanical input power of $\sim 10^{39}$ to 10^{40} erg s⁻¹ for $\sim 10^5$ years. However, there are no BH mass measurements for such ULXs: If they are powered by $\sim 10 M_{\odot}$ BHs, their radiative and/or mechanical power would, in some cases, exceed the Eddington value by a factor of 10, but if they are powered by BHs of mass $> 100 M_{\odot}$ (known as intermediate-mass BHs), they may not exceed the Eddington limit. In the Milky Way, the most energetic BH bubble is probably SS 433/W50, with an average mechanical power of $\sim 10^{39}$ erg s⁻¹ over an age of a few 10^4 years (23–28).

Here, we report the detection of a powerful microquasar in the inner disk of the spiral galaxy M83 (NGC 5236), located at a distance of 4.61 ± 0.26 Mpc (29). We are conducting a multi-wavelength study of x-ray source populations in M83. Our observations include 5.5- and 9.0-GHz images taken with the Australia Telescope Compact Array (ATCA) on 28 to 30 April 2011, for a total observation time of 36 hours. We also used archival data of M83 taken with the Australian Long Baseline Array (LBA) on 13 May 2005, for a total of 9 hours, at 2.3 GHz (30). In the infrared (IR)-optical bands, we used archival Hubble Space Telescope/Wide Field Camera 3 (HST/WFC3) images of the inner disk of the galaxy in several broad- and narrow-band filters (data taken on 20 to 26 August 2009). We observed M83 with the Chandra X-ray Observatory/ACIS-S camera 10 times between December 2010 and December 2011, as part of a large program to study x-ray source populations of a star-forming galaxy; the total exposure time was 729 ks. In addition, we used an archival 50-ks x-ray observation of M83

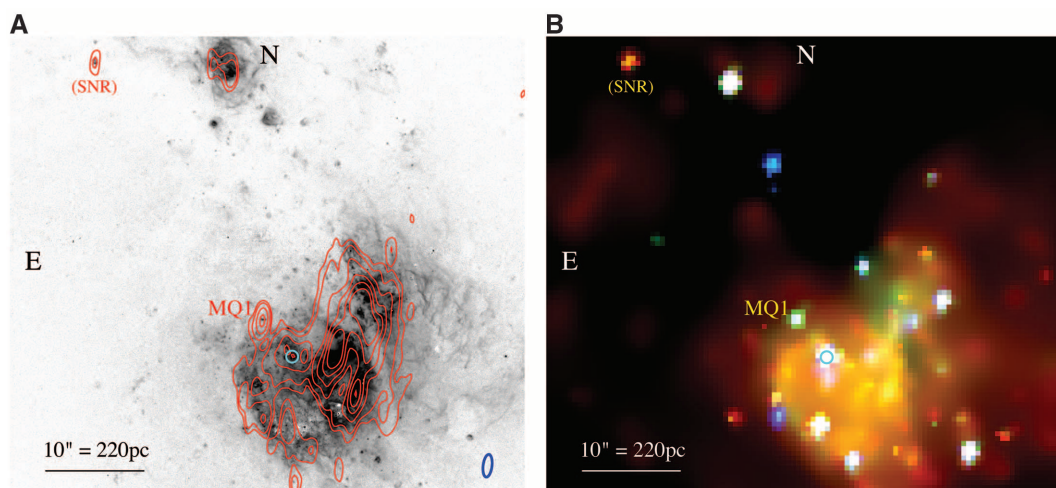
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Fig. 1. Location of MQ1 in the inner region of the M83 galaxy.

(A) HST/WFC3 image of the central region of M83 in the F657N filter, with superposed 9.0-GHz surface brightness contours, from our ATCA data. Contour levels are 0.10, 0.18, 0.40, 0.78, 1.32, and 2.0 mJy per beam. The beam size is $2''.3 \times 0''.9$ at a position angle of 173° . The root mean square noise level in source-free regions of the map is 0.025 mJy per beam, and the peak intensity of MQ1 is 0.9 mJy per beam, but this includes a small contribution from the surrounding extended emission. Labels mark the location of the microquasar MQ1 and of a nearby SNR used for astrometric alignment. The blue circle (radius $0''.6$) marks the nucleus of M83. (B) Chandra/ACIS x-ray color image of the same field (red, 0.35 to 1.1 keV; green, 1.1 to 2.6 keV; blue, 2.6 to 8.0 keV).



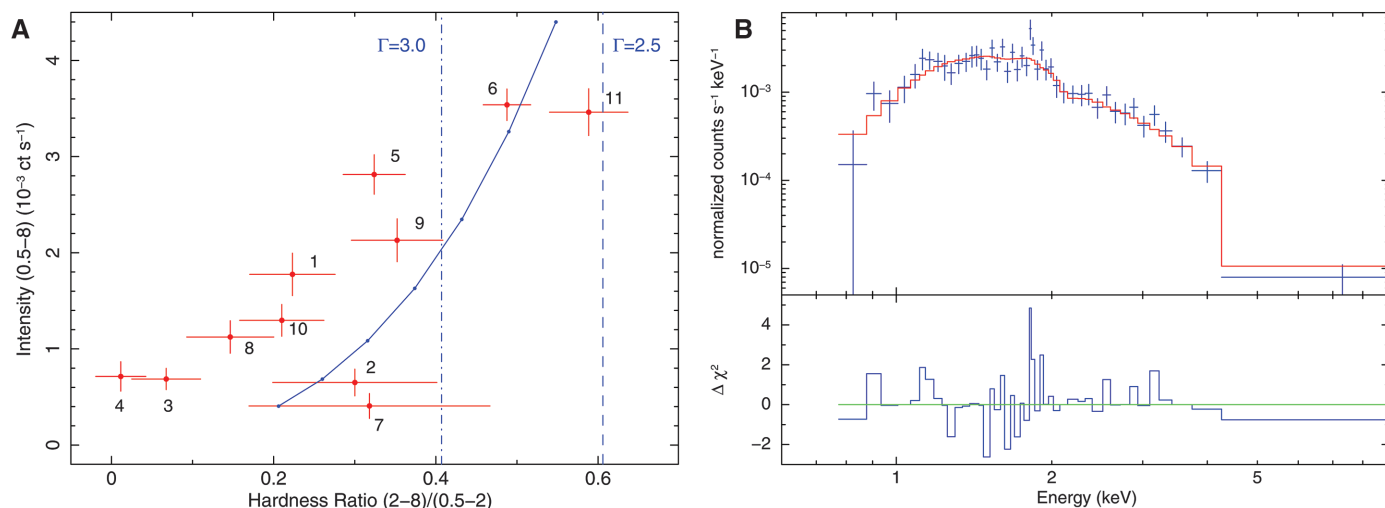


Fig. 2. X-ray hardness and spectral properties. (A) X-ray hardness-versus-intensity diagram of MQ1 from our sequence of Chandra observations. The number next to each data point indicates the order in which the observations were taken (table S1). The blue line denotes the expected location of a disk-blackbody spectrum with temperature $k_B T_{in}$ varying (from top to bottom) from 0.80 to 0.50 keV in steps of 0.05 keV. Dashed and dash-dotted lines mark the

expected hardness of sources with the same absorbing column and power-law photon indices $\Gamma = 2.5$ and 3, respectively: The observed values are clearly inconsistent with the low/hard state ($\Gamma < 2$) at all epochs. ct s^{-1} , counts per second. (B) Combined x-ray spectrum from the Chandra ObsID 12994 (6th observation) and 14342 (11th observation), when the source was brightest. The spectrum is well fitted with an absorbed disk-blackbody model, with parameters typical of stellar-mass BHs (table S3).

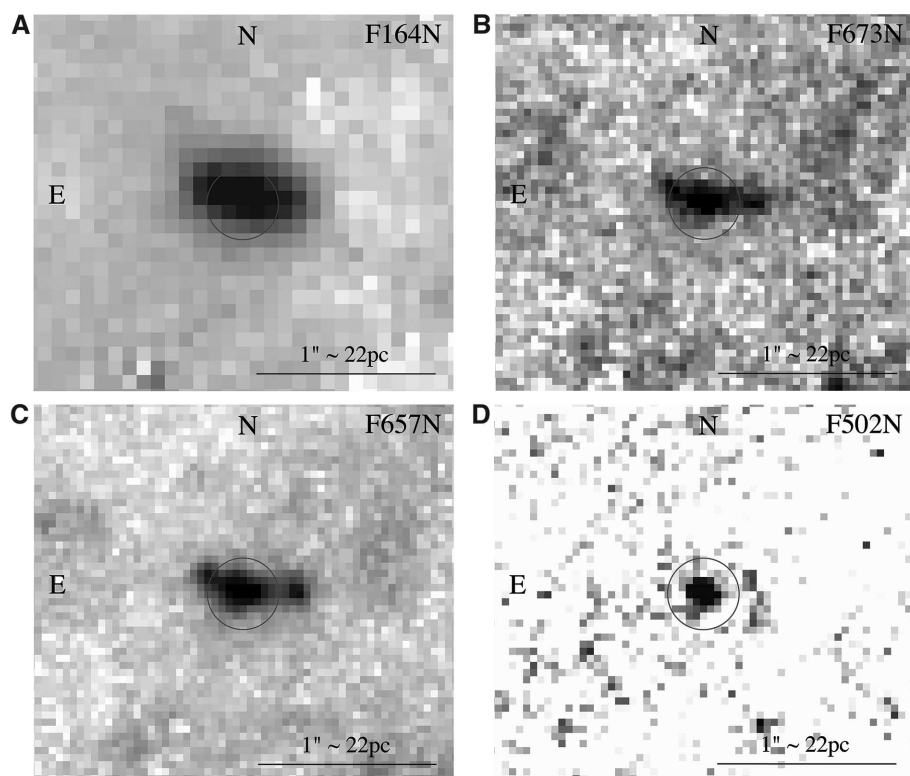


Fig. 3. Optical-IR line emission. (A to D) HST/WFC3 image of the microquasar in four continuum-subtracted bands. Clockwise from top left: [FeII] $\lambda = 1.644 \mu\text{m}$; [SII] $\lambda\lambda 6716, 6731$; [OIII] $\lambda 5007$; and $\text{H}\alpha + [\text{NII}] \lambda\lambda 6548, 6584$. The blue circle indicates the centroid of the ATCA/Chandra source and has a radius of $0''.2$.

obtained with Chandra/ACIS-S in April 2000 (31, 32).

Our ATCA images show a luminous radio source at right ascension (RA) = 13 hours 37 min

1.123 s (± 0.005 s), Dec. = $-29^\circ 51' 52''.17$ ($\pm 0''.10$) (J2000), $\sim 5''$ northeast of the optical nucleus of M83 and just outside the intensely star-forming region that surrounds it (Fig. 1 and fig. S1). It has

a flux density $S_{5.5} = 1.4 \pm 0.1$ millijansky (mJy) and $S_{9.0} = 0.80 \pm 0.05$ mJy at a frequency (ν) of 5.5 and 9.0 GHz, respectively, which corresponds to a radio luminosity $\approx 2 \times 10^{35} \text{ erg s}^{-1}$, similar to those of the two most powerful microquasar bubbles known to date, IC 342 X-1 and NGC 7793-S26 (19, 20, 22). At 5 GHz, this radio source is ≈ 30 times more radio-luminous than SS 433/W50 (24, 26, 27). Henceforth, we identify this source as MQ1. Its steep spectral index $\alpha \approx 1.1$ ($S_\nu \sim \nu^{-\alpha}$) implies optically thin synchrotron emission, possibly from an aging population of electrons. MQ1 is unresolved in the ATCA maps, implying a diameter $\leq 1'' \approx 22$ pc. In the LBA map, an unresolved source (size ≤ 2 pc) is detected at RA = 13 hours 37 min 1.136 s (± 0.003 s), Dec. = $-29^\circ 51' 52''.15$ ($\pm 0''.02$) (J2000) with a flux density of $S_{2.3} = 0.22 \pm 0.04$ mJy at 2.3 GHz, a factor of ≈ 10 to 15 times less than the total expected flux density at that frequency, based on the ATCA data. This indicates that $\sim 90\%$ of the emission is extended over a region of at least a few parsecs in diameter (much larger than the LBA beam but smaller than the ATCA beam). The LBA source is offset to the east of the ATCA centroid by $\approx 0''.2 \pm 0''.1$ (fig. S2).

We interpret the radio source as emission from a radio bubble or radio lobes (that is, from an expanding structure of shocked plasma) inflated by the mechanical power of the BH; synchrotron-emitting electrons are produced where the jet heads hit the interstellar medium (ISM). Similar structures have been found in other non-nuclear BHs: notably, the ring around Cyg X-1 (33), the “ears” of SS 433 (34), and the bubbles around several ULXs (35). We speculate that the LBA radio source arises from the eastern hot spot, where the head of a jet strikes the ISM. The

nondetection of a compact core in the LBA (flux density $S_{2.3} \lesssim 0.1$ mJy per beam) does not imply that the jet is currently turned off, as we estimate [scaling from the power and luminosity of galactic microquasars, e.g., (36)] that the radio flux density of the MQ1 core would always be less than or equivalent to a few microjansky, even for an efficient jet production at high accretion rates.

Our Chandra study shows a luminous point-like source, coincident with MQ1 to within the x-ray positional uncertainty of $0''.3$, whose characteristics provide an estimate of the BH mass. The x-ray flux and hardness vary from one observation to another (Fig. 2 and fig. S3) and also within observations (fig. S4). This variability rules out emission from shocked gas; the source must be an accreting compact object. The x-ray spectrum is always consistent with thermal disk emission (2); it is never consistent with the power-law-dominated low/hard state. When the source is brightest [Chandra X-ray Observatory identification number (ObsID) 12994 and ObsID 14342], a standard disk-blackbody model fit [$tbabs \times tbabs \times diskbb$ in XSPEC version 12.6.0 (37)] gives a characteristic peak temperature $k_B T_{in} \approx 0.7$ keV (where k_B is the Boltzmann constant), apparent inner-disk radius $r_{in} (\cos\theta)^{1/2} \approx 50$ km (where θ is the viewing angle: face-on for $\theta = 0$), and unabsorbed 0.5- to 8-keV luminosity $\approx 7.4 \times 10^{37} (\cos\theta)^{-1} \text{ erg s}^{-1}$. The inferred BH mass depends on the viewing angle and the BH spin: $M \approx (1.19c^2/G)(1/\alpha)r_{in}$ (here, c is the speed of light and G is the gravitational constant) (38), where $\alpha \equiv (c^2/G)(R_{isco}/M) = 6$ for a nonrotating BH and $1.24 < \alpha < 6$ for astrophysically plausible spinning BHs (39, 40), and R_{isco} is the innermost stable circular orbit. From our standard disk-blackbody fit, we obtain $M \approx (42.6_{-9.3}^{+12.8})(1/\alpha)(\cos\theta)^{-1/2} M_\odot$. Thus, for arbitrary midrange values ($\cos\theta \sim 0.5$, Kerr parameter $a \sim 0.5$, implying $\alpha \sim 4$), the spectral model, fitted temperature, inner-disk radius, and luminosity are similar to those found in galactic stellar-mass BHs ($M \sim 10 M_\odot$) accreting at $\sim 10\%$ of the Eddington rate, which supports the stellar-mass BH identification for MQ1 (Occam's razor). For the most extreme (less likely) choice of parameters in their physically acceptable range ($\theta = 85^\circ$, $a \approx 0.998$), the best-fitting BH mass estimate is $M \approx 115 M_\odot$.

There is an optical emission line source at the position of MQ1 that was previously identified with HST (41) and classified as a supernova remnant (SNR) within the nuclear region. Closer inspection of the optical source in the continuum-subtracted H α + [NII] $\lambda\lambda 6548, 6584$, [FeII] $\lambda 1.644 \mu\text{m}$, and [SII] $\lambda\lambda 6716, 6731 \text{ \AA}$ images (Fig. 3) shows that it is resolved into a stronger central source with two knots or lobes at either side (a structure reminiscent of SS 433/W50). The sum of the projected distances between the side knots and the central peak is $\approx 0''.6 \approx 13$ pc. There are no other bright stars or sources of optical continuum emission at this location (fig. S5); thus, the extended optical source is a physical

structure rather than a random alignment of three or more unrelated emission line objects. We used nearby sources detected in more than one band to refine the relative astrometry of the HST image with respect to the radio coordinates, and we conclude that the optical core, the ATCA centroid, and the x-ray position are coincident within $\approx 0''.15$. The eastern knot of H α emission may be associated with the compact LBA source (fig. S2). We suggest that the optical structure represents the core and hot spots and/or lobes of the microquasar.

Optical line emission from the shocked gas is a reliable proxy for mechanical power. For a fully radiative shock expanding into the ISM with velocity v_s , the total radiative flux equals the flux of mechanical energy through the shock (35, 42). Standard bubble theory (43) shows that the bolometric photon luminosity L_T is related to the long-term-averaged mechanical power as $L_T = (27/77)P_J$, with the rest of the power going into the kinetic energy of the expanding shell of swept-up gas and into the thermal energy of the shocked gas between the reverse shock and the swept-up shell. The fraction of bolometric radiative luminosity emitted in a given line (including both the shock and the precursor contributions) can be computed from shock- and photoionization codes such as Modeling And Prediction in Photo-Ionized Nebulae and Gasdynamic Shocks III (MAPPINGS III) (44).

H β or H α are the lines most often used to estimate microquasar jet power (19). However, in this case both lines (especially H β) are affected by high optical extinction; the H α flux from HST imagery includes an uncertain but substantial contribution from [NII] $\lambda\lambda 6548, 6584$; and part of the Balmer emission may come from x-ray photoionization due to the central source. Thus, we used instead the flux in the IR line [FeII] $\lambda = 1.644 \mu\text{m}$ as a proxy for the mechanical power. The [FeII] line is a powerful diagnostic tool for shock-heated gas in high-density regions (45–48).

We measured the [FeII] line flux (table S2) from the HST/WFC3 IR-camera image in the F164N filter after subtracting the continuum emission using the F160W image. The apparent (not corrected for extinction) [FeII] luminosity is $\approx 1.1 \times 10^{37} \text{ erg s}^{-1}$, one order of magnitude greater than that observed from SNRs in the spiral arms. We adopted an extinction $A_V \approx 3.9$ magnitude (mag), as inferred from the x-ray column density [$n_H = (8.6_{-1.5}^{+1.8}) \times 10^{21} \text{ cm}^{-2}$] using the conversion relation of (49), with a likely uncertainty range $A_V \sim 3.8$ to 5 mag. Taking into account the corresponding IR extinction at $1.64 \mu\text{m}$ ($A_{1.64\mu\text{m}} \approx 0.70$ mag, the emitted luminosity $L_{[\text{FeII}]} = (2.1 \pm 0.2) \times 10^{37} \text{ erg s}^{-1}$ (table S2).

To estimate the mechanical power, we assume a preshock atomic number density $n_0 \sim 100 \text{ cm}^{-3}$, typical of the inner disk–nuclear region of M83 (41, 50, 51). Then, from MAPPINGS III models (44), and assuming equipartition between magnetic and gas pressure, we derive that $L_{[\text{FeII}]} = (0.7 \pm 0.1) \times 10^{-3} P_J$ over a wide range of plausible shock velocities (fig. S6). We conclude that $P_J \approx 3 \times 10^{40} \text{ erg s}^{-1} > L_{\text{Edd}}$ (from the BH mass

constraints). We can derive other physical parameters using a well-known self-similar expansion solution for jet-driven bubbles and lobes (43, 52). The characteristic radius R (distance between core and hot spots) scales as $R \approx 0.76 P_J^{1/5} t^{3/5} \rho_0^{-1/5}$ where $\rho_0 \approx \mu m_p n_0$ (m_p is the proton mass and μ the mean atomic weight). Taking $P_J \approx 3 \times 10^{40} \text{ erg s}^{-1}$, $R \approx 0''.3 \approx 6.7$ pc (neglecting projection effects), and $\mu = 1.38$, we infer a characteristic expansion age $t \approx 4.9 \times 10^{11} \text{ s} \approx 16,000$ years. The expansion velocity $v_s = dR/dt = (3/5)R/t \approx 250 \text{ km s}^{-1}$. Taking $n_0 \sim 50$ to 200 cm^{-3} does not substantially change the power but gives an age range $\sim 13,000$ to $20,000$ years and a velocity range $v_s \sim 200$ to 310 km s^{-1} . Mechanical power and shock velocity are similar to those measured in the powerful microquasar NGC 7793 S26 (19, 20, 53), but the age of MQ1 is a factor of 10 younger and the ISM density two orders of magnitude higher.

Both the optical-IR and the radio luminosity come from the bubble and/or lobes; therefore, they trace the time-averaged mechanical power. The x-ray luminosity traces the current accretion rate. It is likely that during the Chandra observations, the system had a sub-Eddington luminosity and a weak jet or none at all. It is also likely that in the past, the system had super-Eddington luminosity and jet power: It would have looked like ULXs such as Holmberg IX X-1 or NGC 1313 X-2 (luminosity $L_X \approx 2 \times 10^{40} \text{ erg s}^{-1}$), surrounded by ionized bubbles with mechanical power $P_J \sim 10^{40} \text{ erg s}^{-1}$ (35). If the x-ray core switches off or fades, the surrounding radio-optical bubble remains bright.

For MQ1, we have been able to constrain the BH mass from x-ray spectral analysis because the BH is currently in a disk-dominated state. None of the other microquasars with similar mechanical power has a reliable constraint on its BH mass, because none of them has been observed in this state. Combined with an accurate determination of time-averaged mechanical power from shock-ionized line luminosities, this enables a robust estimation of the (mechanical) Eddington ratio. Thus, we have identified a system that has had (on average) a super-Eddington mechanical power for $\sim 2 \times 10^4$ years (most likely, a few times above Eddington). MQ1 provides empirical evidence that BHs can sustain super-Eddington mechanical power for an extended period of time. During those phases, BHs inject more energy into the surrounding medium than would be inferred on the basis of their Eddington limits. The existence of super-Eddington mechanical power is important for the modelling of jet production mechanisms, as well as the evolution of fast-growing supermassive BHs in the early universe and their effect on their host galaxies.

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Supplementary Materials

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Supplementary Text
Figs. S1 to S6
Tables S1 to S5
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Large-Amplitude Spin Dynamics Driven by a THz Pulse in Resonance with an Electromagnon

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Multiferroics have attracted strong interest for potential applications where electric fields control magnetic order. The ultimate speed of control via magnetoelectric coupling, however, remains largely unexplored. Here, we report an experiment in which we drove spin dynamics in multiferroic TbMnO₃ with an intense few-cycle terahertz (THz) light pulse tuned to resonance with an electromagnon, an electric-dipole active spin excitation. We observed the resulting spin motion using time-resolved resonant soft x-ray diffraction. Our results show that it is possible to directly manipulate atomic-scale magnetic structures with the electric field of light on a sub-picosecond time scale.

Data storage devices based on ferromagnetic or ferroelectric materials depend strongly on domain reorientation, a process that typically occurs over time scales of several nanoseconds. Faster reorientation dynamics may be achievable by using intense

electromagnetic (EM) pulses (*1*). The EM pulses can couple to magnetism either indirectly via electronic excitations (*2*) or directly via the Zeeman torque induced by the magnetic field (*3–5*). Direct excitation has the advantage of minimal excess heat deposition but requires

frequencies in the 10¹⁰ to 10¹² Hz range. The low magnetic field strength of currently realizable THz-frequency EM sources poses a formidable challenge for such schemes.

Thanks to the coexistence of different ferroic orders, multiferroics offer new routes to domain control (*6*). Particularly strong coupling between the ferroelectric and magnetic order exists in single-phase frustrated magnets, where noncollinear spin structure drives ferroelectricity as a result of weak relativistic interactions (*7–9*). Consequently, the magnetic order can be controlled by application of an electric field (*10–13*).

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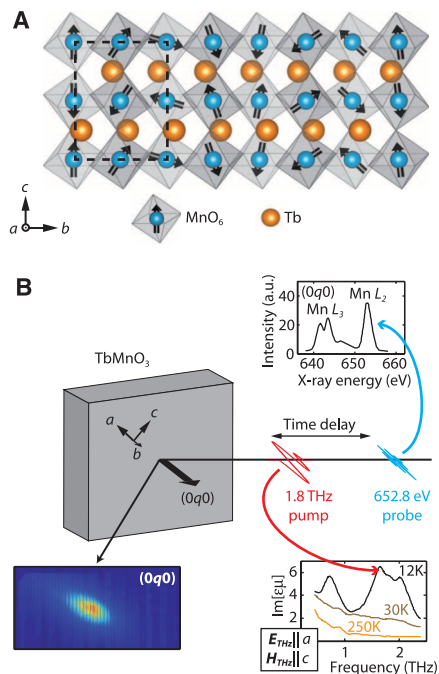


Fig. 1. Experimental setup. (A) The magnetic structure of TbMnO₃ below 27 K. The spins of the Mn 3d shells (black arrows) form a cycloid propagating within the (*bc*) crystallographic plane. The oxygens are represented by gray octahedra around the Mn atoms (blue spheres). The black dashed box indicates a unit cell. (B) Schematic of the experiment. A THz pulse resonant with the strongest electromagnon [lower right inset, spectrum measured with orientation of electric (E_{THz}) and magnetic (H_{THz}) field of THz denoted in the box (26)] excites spin motion in the sample. An x-ray pulse resonant with the Mn L_2 edge (upper inset) measures the response as changes in the intensity of the (0 q 0) diffraction peak (lower left inset). a.u., arbitrary units.

However, the speed of domain switching triggered by simple step-function-like electric fields appears to be limited to a time scale of several milliseconds (14). As an alternate solution, optical pulses have been shown to affect the magnetic structure of multiferroics on a femto- and picosecond time scale (15–17). It has been predicted that ultrafast magnetic dynamics can be also triggered by coherent excitation of electromagnons, electric-dipole active spin excitations directly connected to the magnetoelectric coupling (18). Here, we show experimentally that a few-cycle THz pulse tuned to resonance with an electromagnon can transiently modify the magnetic structure of multiferroic TbMnO₃.

TbMnO₃ is a model spin-cycloid multiferroic exhibiting strong magnetoelectric coupling. Although it has a relatively simple perovskite atomic structure, a strong GdFeO₃-type distortion gives rise to a variety of spin-frustrated phases (19, 20). At room temperature, the crystal is paramagnetic. Below 42 K, the Mn spins form a paraelectric sinusoidally modulated spin density wave (SDW) state, which transforms into a spin-

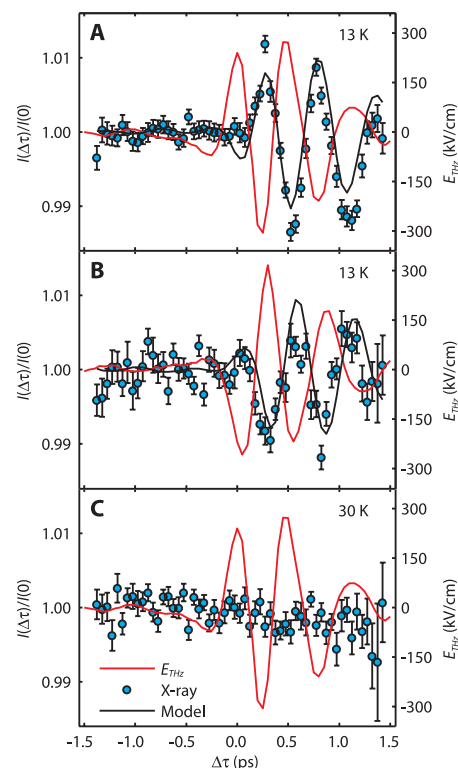


Fig. 2. Time-dependent behavior. The magnetic diffraction intensity I of the (0 q 0) peak of TbMnO₃ (blue symbols, left axis) compared with the electric field E_{THz} of the pump trace (red solid line, right axis) as a function of the time delay. (A and B) The response of the crystal in the multiferroic phase ($T = 13$ K) for opposite signs of the driving electric field. The solid black lines are based on a model discussed in the text. (C) The response in the SDW phase ($T = 30$ K). (D) Fourier transform of the THz and x-ray traces from (A). Error bars indicate a standard error for every point (34).

cycloid state below 27 K. In this phase, the spins form a cycloid within the (*bc*) crystallographic plane (Fig. 1A), and a spontaneous ferroelectric polarization along the *c* axis develops. Microscopically, the spin current between canted spins on neighboring sites i and j gives rise to a ferroelectric polarization $\vec{p}_{ij} \propto \vec{r}_{ij} \times (\vec{S}_i \times \vec{S}_j)$ (7), and the magnitude of the polarization is further enhanced by lattice displacements (21–23). In all these spin-frustrated phases, the magnetic structure of the Mn spins is incommensurate with the lattice, characterized by a wave vector $\mathbf{k} = (0, q, 0)$, where $q \approx 0.28$ changes very slowly in the SDW phase with temperature (24).

The EM excitation spectrum of TbMnO₃ shows broad peaks in the THz frequency range; these have been assigned to electromagnons (25–29) (Fig. 1B, lower inset). The strongest feature at 1.8 THz is activated with the electric component of light parallel to the *a* axis and is absent in other geometries (26). It has been proposed that the oscillating electric field along the *a* axis modifies the nearest neighbor ferromagnetic exchange constant in the (*ab*) plane, resulting in antiphase spin oscillations within the spin-cycloid plane (28, 30). Weaker spectral features at lower frequencies have been proposed to arise from the higher harmonics of the spin cycloid and coupling to the strongest electromagnon (30) and from out-of-plane spin-cycloid motions (28, 31, 32).

To investigate whether excitation of electromagnons in TbMnO₃ is a viable route for magnetic order control, we performed a THz pump and soft x-ray probe experiment (Fig. 1B). The

sample is a single crystal of TbMnO₃ cut to the (010) surface, oriented so that the *a* axis is at 45° with respect to the horizontal scattering plane. We generated few-cycle, phase-stable THz pulses with a center frequency of 1.8 THz by using optical rectification in a nonlinear organic crystal with a peak electric field of about 300 kV/cm at focus (33). We measured the electric field component of the THz waveform at the sample position by using electrooptic sampling. To see the spin motion resulting from the excitation, we used time-resolved resonant soft x-ray diffraction at the Mn L_2 edge and measured the intensity of the first-order (0 q 0) cycloid reflection (34).

The spin dynamics can be extracted from the behavior of the intensity of the (0 q 0) diffraction peak as a function of pump-probe delay time, $\Delta\tau$ (Fig. 2). At $T = 13$ K, where TbMnO₃ is deep in the multiferroic phase, the x-ray signal shows oscillations resembling the shape of the THz pump-pulse electric field (Fig. 2A). The observed modulation of the diffraction peak intensity is over an order of magnitude larger than expected for unconstrained spin precession driven directly by the magnetic field component of the THz pulse (34). The Fourier transform of the x-ray trace (Fig. 2D) shows that the material response has essentially the same frequency spectrum as both the pump and the electromagnon. The delay between the first maximum of the pump trace and the first maximum of the x-ray trace is 250 fs, corresponding to about half of a single oscillation cycle. Inverting the sign of the electric field of the pump pulse results in an opposite

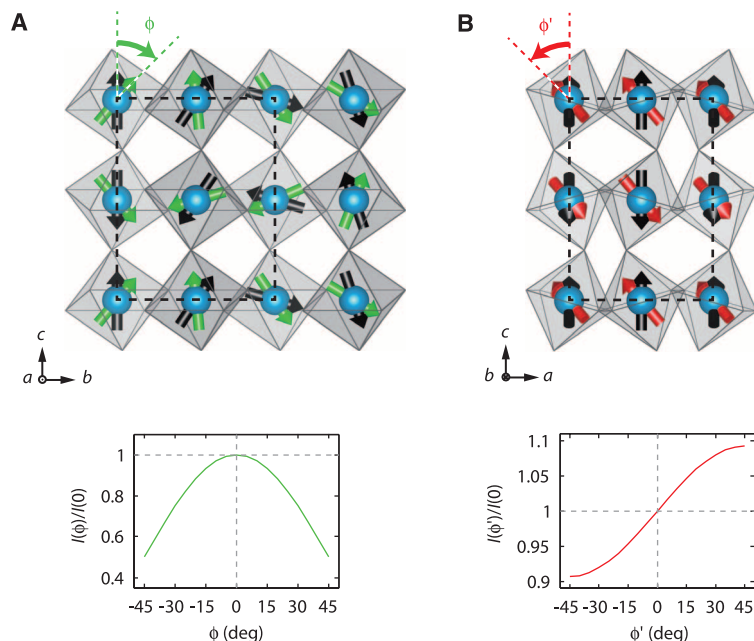
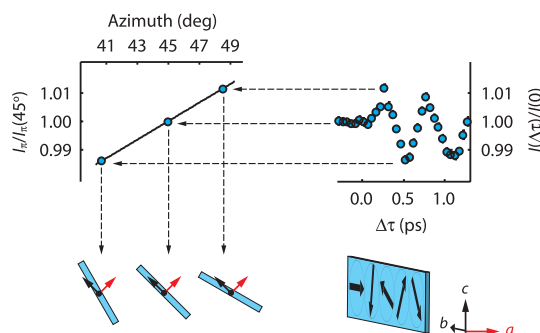


Fig. 3. Spin-motion patterns analyzed to interpret the time-dependent data. The illustrations depict the different patterns of how the magnetic structure changes. Black arrows denote how the spins are oriented in the ground state. Color arrows indicate the spin directions at one of the extremes of the excited motion. Tb ions have been removed for clarity. The graphs show calculations of the changes in $(0q0)$ peak intensity as a function of the motion coordinate. (A) Antiphase oscillation within the spin-cycloid plane, parameterized by using the spin rotation coordinate ϕ and viewed along the a axis. (B) Coherent rotation of the spin-cycloid plane by an angle ϕ' about the crystallographic b axis viewed along the b axis.

Fig. 4. The diffracted intensity versus spin-cycloid rotations. (Left) Azimuthal dependence of the $(0q0)$ peak for the π -polarized incident x-rays divided by the diffracted intensity at an azimuth of 45° . (Right) Time-resolved diffracted intensity normalized to the intensity before excitation. The blue plane represents a plane of a single spin cycloid propagating along the crystallographic b axis. The a axis is marked with red. The angles of rotation in the drawing have been exaggerated for clarity.



sign of changes in the diffraction intensity transients (Fig. 2B). Such behavior is expected when it is the electric field, and not simple heating, that drives the spin motion. When TbMnO_3 is in the non-multiferroic SDW phase ($T = 30$ K), the oscillation in the peak intensity after the pump is strongly suppressed (Fig. 2C). This temperature dependence gives strong evidence that the THz-induced spin motion is correlated with the presence of multiferroicity. At 30 K, we tentatively attribute the slight drop of overall intensity after the pump to heating effects from absorption of the THz pulse, which leads to an estimated temperature increase of less than 0.05 K (34).

To better understand the time dependence of the spin response, we construct a very simple model of the system as two independent simple harmonic oscillators at the electromagnon

resonance frequencies of 0.7 and 1.8 THz (34). Although not a perfect match to the data, the behavior of the conjugate momentum of the higher-frequency oscillator successfully reproduces the general shape of the oscillation and the delay between the driving electric field and the changes in x-ray diffraction (Fig. 2, A and B). The agreement is much worse for either canonical coordinate of the lower-frequency oscillator, suggesting that off-resonant excitation of the lower-energy electromagnon or other purely magnetic modes is not consistent with the measured shape or delay of the response (34).

Resonant x-ray scattering at the Mn L edge is predominantly sensitive to the magnetic moment of the Mn 3d shell (35). An analysis of how different spin motions contribute to the intensity of the diffraction peak allows us to test which of

them are involved in the observed oscillations. We consider two components of the induced spin motion, motivated by the current understanding of spin dynamics in this system. In the first component, the spins move in antiphase within the spin-cycloid plane, the pattern widely considered to be responsible for the infrared activity of the 1.8-THz electromagnon (28). The driving electric field applies an effective “force” to this component. In our model of the electromagnon as a harmonic oscillator, this component of the spin motion should then be identified with the “position” of the oscillator. In the proposed pure spin Hamiltonian for this system (18), the conjugate momentum must be a spin motion orthogonal to this position coordinate. Numerical simulations based on this Hamiltonian have predicted that a sufficiently intense THz pulse in resonance with the 1.8-THz electromagnon can induce coherent rotation of the spin-cycloid plane about the b axis until it reaches another stable orientation in either the (ab) or the (bc) plane (18). In our experiment, the effective THz pulse field strength is over two orders of magnitude lower than that used in these simulations (34), and so we do not expect to see a persistent domain reorientation. Instead, we propose to consider a smaller rotation of the spin-cycloid plane about the b axis as a second component of the spin motion that corresponds to the conjugate momentum for the 1.8-THz resonance.

We model these two spin-motion patterns separately as distortions to the equilibrium magnetic structure that influence the magnetic structure factor (34). We then calculate the intensity of the $(0q0)$ diffraction peak as a function of each coordinate of the spin motion (Fig. 3). For the in-plane motion, the intensity of the diffraction peak is an even function of the spin coordinate, giving a decrease of the diffracted intensity with twice the frequency of the spin motion (Fig. 3A). For the spin-cycloid plane rotation, the change of diffracted intensity is an odd function of the rotation angle. This motion then leads to a modulation of the diffraction intensity with the same frequency as the spin motion (Fig. 3B). For a field-driven excitation process, we expect the spin-motion frequency to be the same as the frequency of THz pump. We conclude that the main motion visible in our experiment is a rotation of the spin-cycloid plane. The in-plane spin motion may also be present, but its response would be suppressed at the current experimental time resolution. This is consistent with our harmonic oscillator model, which suggests that we see primarily dynamics of the conjugate momentum of the resonance.

For π -polarized x-rays at the Mn L_2 edge, the scattering intensity is a strongly varying function of the sample azimuth (rotation about the Bragg wave vector) (34). Rotation of the spin-cycloid plane about the b axis induced by the THz pulse is equivalent to rotating the sample about the $(0q0)$ scattering vector. Hence, we interpret the data quantitatively by comparing the

change of the intensity of the diffraction peak seen in the pump-probe trace with the change seen upon rotating the sample by a small angle around the azimuth of 45° in equilibrium conditions (Fig. 4). We estimate that the observed $1.35\% \pm 0.12\%$ (SE) maximum change of peak intensity corresponds to an amplitude of spin-cycloid plane rotation equal to $4.2^\circ \pm 0.4^\circ$ (34). We expect that higher fields will lead to larger spin-cycloid rotations. A simple linear extrapolation suggests that THz pulses with an amplitude of 1 to 2 MV/cm inside the sample could lead to spin-cycloid rotations on the order of 90° . We can compare this against the model of (18), which predicts switching at 14 to 15 MV/cm for single-cycle THz pulses.

Our results show that intense THz pulses can modify the magnetic order in a multiferroic. Given that TbMnO_3 is a model compound for a large group of materials with noncollinear spin order, our results serve as a proof of principle for a wide range of compounds. Moreover, the presence of magnetoelectric coupling in multiferroic heterostructures encourages a search for similar mechanisms as a basis for technologically feasible multiferroic devices.

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Supplementary Materials

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Materials and Methods
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Angular Fluctuations of a Multicomponent Order Describe the Pseudogap of $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$

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The hole-doped cuprate high-temperature superconductors enter the pseudogap regime as their superconducting critical temperature, T_c , falls with decreasing hole density. Recent x-ray scattering experiments in $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$ observe incommensurate charge-density wave fluctuations whose strength rises gradually over a wide temperature range above T_c , but then decreases as the temperature is lowered below T_c . We propose a theory in which the superconducting and charge-density wave orders exhibit angular fluctuations in a six-dimensional space. The theory provides a natural quantitative fit to the x-ray data and can be a basis for understanding other characteristics of the pseudogap.

Recent x-ray scattering experiments (1–3) in the cuprate superconductor $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$ have observed the formation of temperature-dependent incommensurate charge-density wave (CDW) fluctuations. In $\text{YBa}_2\text{Cu}_3\text{O}_{6.67}$, the CDW scattering intensity at the incommensurate wave vectors $\mathbf{Q}_x \approx (0.31, 0)$ or $\mathbf{Q}_y \approx (0, 0.31)$ (Fig. 1)

increases gradually below temperature $T \approx 200$ K in a concave-upward shape until just above $T_c = 60$ K. One interpretation is that this represents an order parameter of a broken symmetry, with the correlation length arrested at a finite value by disorder; however, such order parameters invariably have a concave-downward shape. The temperature range is also too wide to represent the precursor critical fluctuations of an ordering transition. Indeed, there are no indications of an ordering transition below T_c , and, notably, the scattering intensity decreases below T_c at a rate similar to the rate of increase above T_c .

Instead, the increase in intensity between 200 and 60 K is reminiscent of a gradual increase in the neutron scattering intensity at the antiferromagnetic wavevector in the insulating antiferromagnet La_2CuO_4 between 550 and 350 K (5–7). This increase was explained by the classical thermal, angular fluctuations of the three-component antiferromagnetic order parameter in $d = 2$ spatial dimensions (8). Indeed, more generally, order parameters with $N \geq 3$ components are dominated by angular fluctuations in $d = 2$ (9); here, we find that the $N = 6$ case describes the x-ray scattering in the pseudogap of $\text{YBa}_2\text{Cu}_3\text{O}_{6.67}$.

Previous work (10) used a Landau theory framework (11) to describe competition between superconductivity and CDW order (12, 13). The Landau theory introduces a complex field, $\Psi(\mathbf{r})$, to represent the superconductivity and two complex fields, $\Phi_{x,y}(\mathbf{r})$, to represent the charge order. The latter can represent modulations at the incommensurate wave vectors $\mathbf{Q}_{x,y}$ in not only the site charge density but also modulations in bond variables associated with a pair of sites (13, 14); nevertheless, we will refer to it simply as “charge” order. The free energy is restricted by three distinct $U(1)$ symmetries: charge conservation, translations in x , and translations in y ; which rotate the phases of Ψ , Φ_x , and Φ_y , respectively. There are also the discrete symmetries of time-reversal and the square lattice point group, and these lead to the following form of the Landau free energy

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density (we ignore possible anisotropies in the spatial derivative terms):

$$F = |\nabla\Psi|^2 + s_1|\Psi|^2 + u_1|\Psi|^4 + |\nabla\Phi_x|^2 + |\nabla\Phi_y|^2 + s_2(|\Phi_x|^2 + |\Phi_y|^2) + u_2(|\Phi_x|^2 + |\Phi_y|^2)^2 + w(|\Phi_x|^4 + |\Phi_y|^4) + v|\Psi|^2(|\Phi_x|^2 + |\Phi_y|^2) \quad (1)$$

here, s_1 and s_2 are couplings that tune the superconducting and charge orders, respectively; and u_1, u_2, w , and v are the allowed quartic couplings. The earlier analysis (10) considered “phase” and “vortex” fluctuations of only the superconducting order, Ψ , and then assumed that the charge-order amplitude was proportional to $-v|\Psi|^2$, where $v > 0$ is the competing order coupling: This analysis found a small decrease in charge order with decreasing T but did not find a prominent peak near T_c . Here, we shall provide a theory that is nonperturbative in v , includes the thermal fluctuations of both Ψ and $\Phi_{x,y}$ self-consistently, and applies over a wide range of temperatures.

Our starting assumption is that it is always preferable for the electronic Fermi surface to locally acquire some type of order, and so the region around the origin of the six-dimensional space defined by (Ψ, Φ_x, Φ_y) should be excluded (Fig. 2). For each radial direction in this six-dimensional space, we can label the optimal state by a unit vector n_α ($\alpha = 1 \dots 6$) with $\Psi \propto n_1 + in_2$, $\Phi_x \propto n_3 + in_4$, and $\Phi_y \propto n_5 + in_6$. We will neglect amplitude fluctuations along the radial direction and focus solely on the angular fluctuations; no assumptions of an approximate $O(6)$ symmetry are made a priori. We introduce a partition function for angular fluctuations of n_α , keeping all the terms allowed by the symmetries noted earlier:

$$\mathcal{Z} = \int Dn_\alpha(r) \delta \left[\sum_{\alpha=1}^6 n_\alpha^2(r) - 1 \right] \exp \left(-\frac{\rho_s}{2T} \int d^2r \times \left\{ \sum_{\alpha=1}^2 (\nabla n_\alpha)^2 + \lambda \sum_{\alpha=3}^6 (\nabla n_\alpha)^2 + g \sum_{\alpha=3}^6 n_\alpha^2 + w \left[(n_3^2 + n_4^2)^2 + (n_5^2 + n_6^2)^2 \right] \right\} \right) \quad (2)$$

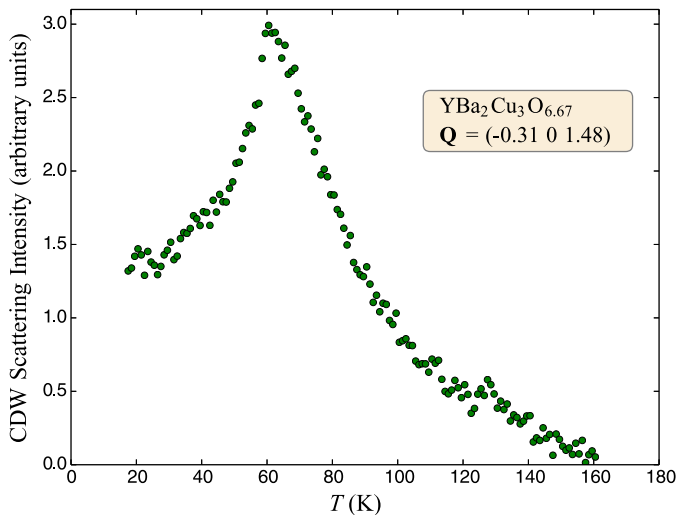


Fig. 1. The temperature dependence of the CDW scattering intensity. The resonant x-ray scattering was measured (4) at $\mathbf{Q} = [-0.31 \ 0 \ 1.48]$ in $\text{YBa}_2\text{Cu}_3\text{O}_{6.67}$. This sample has $T_c \approx 65.5$ K.

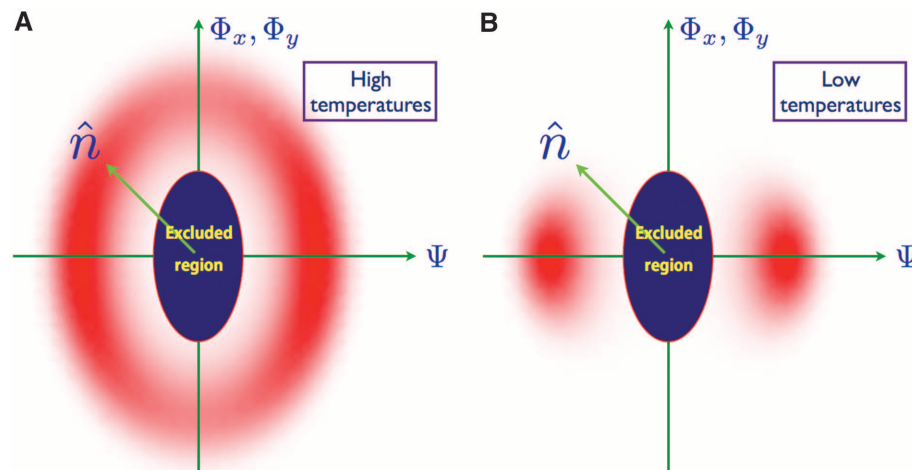


Fig. 2. Schematic of the structure of fluctuations of $\mathcal{Z}$. (A and B) The six-dimensional space represents the complex superconducting order, Ψ , and the complex charge orders $\Phi_{x,y}$. The red shading represents the probability that the values of $\Psi, \Phi_{x,y}$ take particular values. At high T , all angles are explored, whereas at low T below T_c , for $g > 0$, the order lies mainly along the equator in the plane representing Ψ .

The couplings ρ_s and $\rho_s\lambda$ are the helicity moduli for spatial variations of the superconducting and charge orders, respectively. The coupling g determines the relative energetic cost of ordering between the superconducting and charge-order directions; this is the most relevant term that breaks the $O(6)$ symmetry present for $\lambda = 1, g = 0$, and $w = 0$ to $O(4)$ -by- $O(2)$ symmetry. Last, w imposes the square lattice point group symmetry on the charge order: For $w < 0$, the charge is unidirectional with only one of Φ_x or Φ_y nonzero; whereas for $w > 0$, the charge ordering is bidirectional. The final symmetry of $\mathcal{Z}$ is $O(2) \times O(2) \times O(2) \rtimes \mathbb{Z}_2$, where the three $O(2)$'s are enlarged by discrete symmetries from the three $U(1)$'s noted earlier and the $\mathbb{Z}_2$ represents the 90° spatial rotation symmetry, whose spontaneous breaking is measured by the Ising-nematic order (15) $m = |\Phi_x|^2 - |\Phi_y|^2$.

The enhanced symmetries of $\mathcal{Z}$ at $\lambda = 1, g = 0$, and $w = 0$ include two $SO(4)$ rotation symmetries between d -wave superconductivity and incommensurate d -wave bond order that emerge at low energies in the vicinity of a generic quantum critical point for the onset of antiferromagnetism in a metal (16) [but with charge order $\mathbf{Q}$'s along the $(1, \pm 1)$ directions]; a nonlinear sigma model of this theory was developed in (17) and applied to the phase diagram in a magnetic field (18). It was also argued (14) that these symmetries can be viewed as remnants of the $SU(2)$ pseudospin gauge invariances of Mott insulators (19–21) when extended to metals with a strong local antiferromagnetic exchange coupling. We note the similarity to the $SO(5)$ nonlinear sigma model of competing orders (22), which has antiferromagnetism rather than charge order competing with superconductivity.

A crucial feature of our analysis of $\mathcal{Z}$ is that the couplings ρ_s, g, λ , and w are assumed to be T -independent. The dependence on absolute temperature arises only from the Boltzmann $1/T$ factor in $\mathcal{Z}$, and this strongly constrains our fits to the experimental data. This feature ensures our restriction to angular and classical fluctuations in the order parameter space.

We computed the properties of $\mathcal{Z}$ by using a classical Monte Carlo simulation. This was performed by using the Wolff cluster algorithm after the continuum theory was discretized on a square lattice of spacing a . This lattice is not related to the underlying square lattice of Cu atoms in the cuprates; instead, it is just a convenient ultraviolet regularization of the continuum theory, and we do not expect our results to be sensitive to the particular regularization chosen. All length scales in our results will be proportional to the value of a , and the value of a has to be ultimately determined by matching one of them to experiments. We performed simulations on lattice sizes up to 72 by 72 and were able to control all finite size effects.

We also performed a $1/N$ expansion on a generalized model with N components of n_α , as described in (23). It was found to be accurate for the charge-order correlations but does not properly

describe the superconducting correlations near T_c and below.

With the Monte Carlo method, we computed the structure factor

$$S_{\Phi_x}(p) = \int d^2r \sum_{\alpha=3}^4 \langle n_{\alpha}(r) n_{\alpha}(0) \rangle e^{ip \cdot r} \quad (3)$$

In the x-ray experiments, p represents deviations from the wave vectors $\pm Q_x, \pm Q_y$. We show the values of $S_{\Phi_x} \equiv S_{\Phi_x}(p=0)$ for a variety of parameters in Fig. 3. At high T , we have a regime

of increasing S_{Φ_x} with decreasing T , as the correlation lengths of both the superconductivity and the charge order increase and the order parameter fluctuates over all six directions (Fig. 2). At low T , there is onset of superconducting order, and S_{Φ_x} decreases with decreasing T , as the order parameter becomes confined to the Ψ plane. For each set of parameters in Fig. 3, we fit the position of the peak in S_{Φ_x} by choosing the value of ρ_s and adjust the vertical scale so that the peak height also coincides. This amounts to a rescaling (but

not shifting) of the T axis. The peak shape and the ratio of the peak width to the peak position are determined by the theory and can thus be used to fix the values of the dimensionless parameters ga^2 , wa^2 , and λ . The theory naturally reproduces the experimental curve, including the rate of decrease of charge order on both sides of the peak, for a range of parameter values.

There are differences between the experiment and theory in Fig. 3, both at very low and very high T . However, the deviations are in the expected

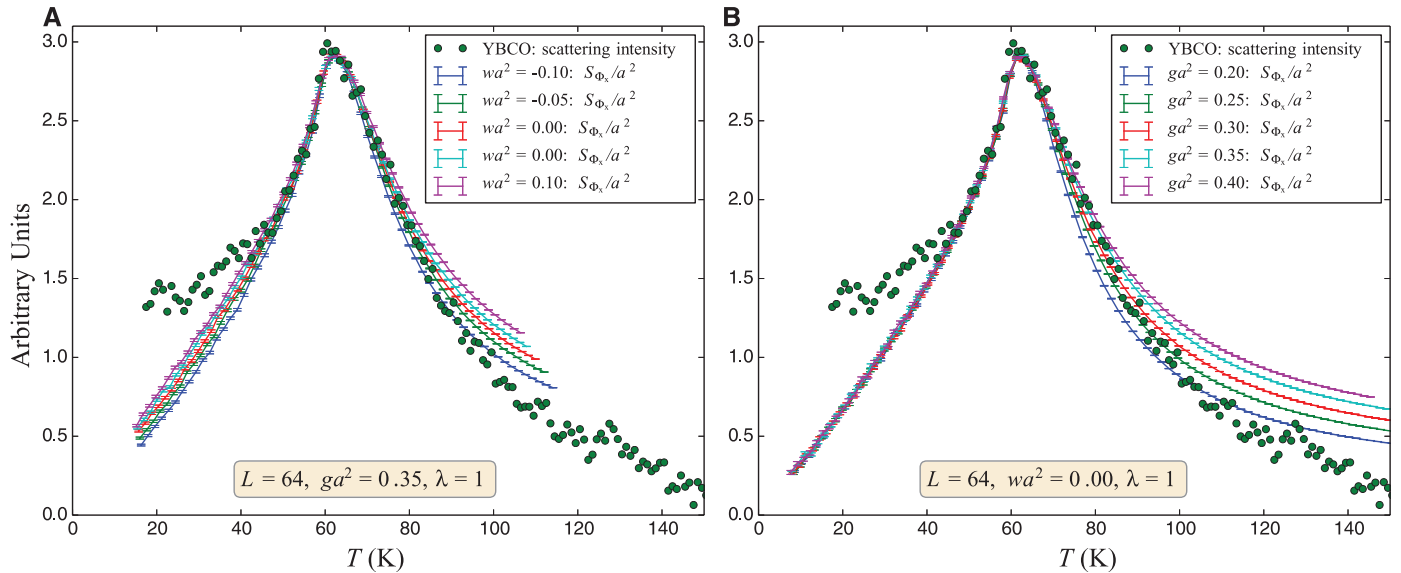


Fig. 3. Comparison of x-ray data for the CDW scattering intensity to S_{Φ_x} calculated in Monte Carlo simulations of Z . The experimental data are as in Fig. 1. (A and B) For each set of values of ga^2 , wa^2 , and λ , there were two fitting parameters: The value of ρ_s was determined for each parameter set so that the peak positions match, and the heights of the Monte Carlo curves

were rescaled to make the peak heights match. The determination of ρ_s is equivalent to a rescaling (but not shifting) of the T axis but does not determine the peak shape; the peak width was not a fitting parameter. For $ga^2 = 0.30$ and $wa^2 = 0.0$, we have $\rho_s = 160$ K. Absolute values of S_{Φ_x} with no rescaling appear in Fig. 4 and (23).

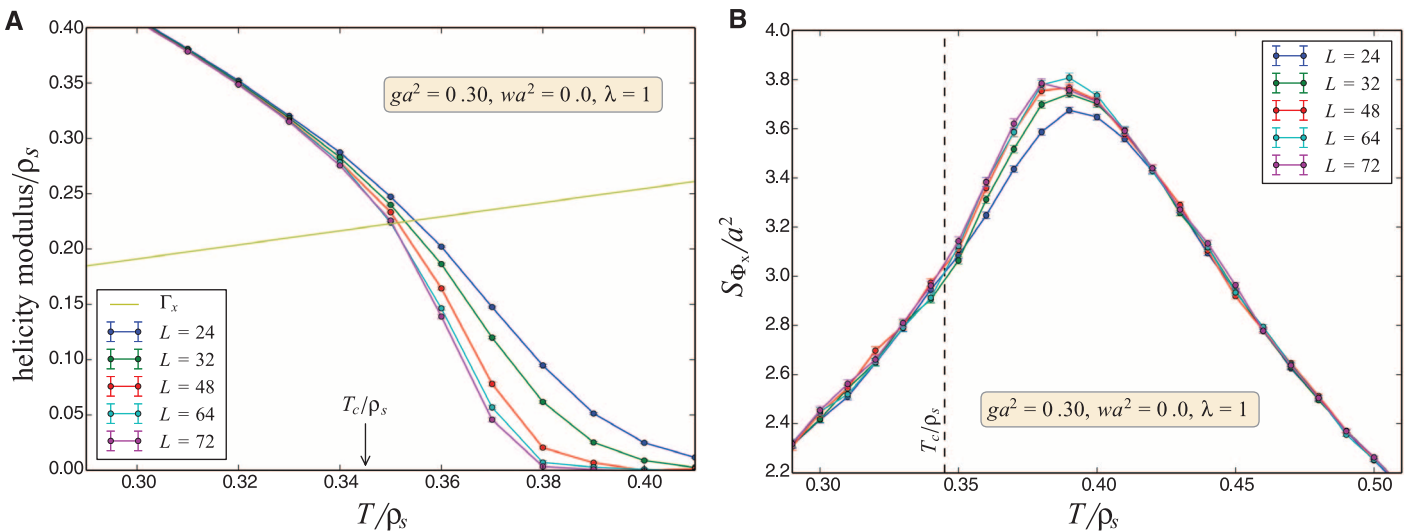


Fig. 4. Monte Carlo results for the helicity modulus and the structure factor. All axes measure dimensionless quantities. (A) Helicity modulus measured in the x direction. Note that ρ_s is the helicity modulus at $T = 0$. The yellow line representing $\Gamma_x = (2\pi)/\rho_s$ is used with the relation helicity modulus $= (2\pi)/T_c$ (26) to determine the Kosterlitz-Thouless temperature for each

system size L . A finite-size scaling analysis estimates $T_c/\rho_s \approx 0.345$ for these parameters. (B) The structure factor, showing a peak at around $T/\rho_s = 0.39$. The Kosterlitz-Thouless temperature, T_c , is marked with a vertical dashed line. The prediction of (10) of increasing charge order with increasing temperature applies in the immediate vicinity of T_c to the left of the peak.

directions. At low T , in the present classical theory without randomness, S_{Φ_x} vanishes as $T \rightarrow 0$; however, pinning of the charge order by impurities is likely responsible for the observed S_{Φ_x} (24, 25). At high T , our assumption of a T -independent bare ρ_s starts failing at $T \approx 2T_c$, and we expect a crossover to a theory with substantial amplitude fluctuations and smaller S_{Φ_x} with increasing T .

Next, we examined the superconducting correlations by computing the associated helicity modulus (Fig. 4). This allows us to determine T_c by comparing against the expected universal jump (26). We find a T_c below the peak in S_{Φ_x} . This is consistent with the arguments in (10), which predicted a monotonic decrease in charge order through T_c : Evidently those computations only apply in a narrow window about T_c . We have not accounted for interlayer couplings in our two-dimensional theory: These can raise T_c and yield a cusplike singularity in the charge order at T_c .

One of the fundamental aspects of our theory is that the same set of parameters used above to describe x-ray scattering experiments also predict the strength of superconducting fluctuations above T_c . The latter are detectable in diamagnetism measurements, and indeed $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$ shows substantial fluctuation diamagnetism (27, 28) over the range of temperatures that x-ray experiments measure charge-order fluctuations. We compute the diamagnetic susceptibility in the $N = \infty$ theory (23). Such a theory has effectively Gaussian superconducting fluctuations, albeit with a T dependence of the superconducting coherence length, which is different from the standard Landau-Ginzburg form (29). An absolute comparison of this theory with the observations (28) yields the value of a , which is found to differ by about 33% from the value obtained from the charge-order correlation length. Considering the simplicity of the $N = \infty$ theory, the possible differences in the x-ray and diamagnetism samples, and the absence of fitting to determine λ and w , this result is encouraging. For a sharper comparison, a Monte Carlo study of the crossover into a vortex-dominated regime (30–32) is needed. Eventually, with a complete study that also includes the effects of disorder and more detailed measurements of charge order and superconducting correlations on the same sample, we expect to be able to more tightly constrain the values of ga^2 , wa^2 , λ , and a .

Although we have only applied the theory to a doping where charge order is most pronounced, we argue that it is characteristic of the entire pseudogap phase. The dominant paradigms for the pseudogap have been phase-fluctuating superconductivity (33) and competing order (12, 13), with experiments providing merit to both descriptions (1–3, 34–36). This work unifies these paradigms in a single multicomponent order parameter that provides a natural description of the x-ray and diamagnetism data. Computations of the influence of fluctuating superconductivity on photoemission spectra (37) should now be extended to include all components of our order parameter. Our model is also linked to theories of metals with antiferro-

magnetic spin fluctuations (16, 17): With decreasing doping, there is a zero-field quantum critical point to the onset of antiferromagnetic order (38), and this indicates that our present model will have to be extended to explicitly include spin fluctuations (39) to apply at such densities.

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Highly Crystalline Multimetallic Nanoframes with Three-Dimensional Electrocatalytic Surfaces

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Control of structure at the atomic level can precisely and effectively tune catalytic properties of materials, enabling enhancement in both activity and durability. We synthesized a highly active and durable class of electrocatalysts by exploiting the structural evolution of platinum-nickel (Pt-Ni) bimetallic nanocrystals. The starting material, crystalline PtNi₃ polyhedra, transforms in solution by interior erosion into Pt₃Ni nanoframes with surfaces that offer three-dimensional molecular accessibility. The edges of the Pt-rich PtNi₃ polyhedra are maintained in the final Pt₃Ni nanoframes. Both the interior and exterior catalytic surfaces of this open-framework structure are composed of the nanosegregated Pt-skin structure, which exhibits enhanced oxygen reduction reaction (ORR) activity. The Pt₃Ni nanoframe catalysts achieved a factor of 36 enhancement in mass activity and a factor of 22 enhancement in specific activity, respectively, for this reaction (relative to state-of-the-art platinum-carbon catalysts) during prolonged exposure to reaction conditions.

Platinum (Pt) is a highly efficient electrocatalyst for both the cathodic oxygen reduction reaction (ORR) in fuel cells (and metal-air batteries) and the hydrogen evolution reaction

(HER) in alkaline electrolyzers (1–19). However, the high cost and scarcity of Pt are key obstacles for its broad deployment in fuel cells and metal-air batteries for both stationary and portable

applications. Intense research efforts have been focused on developing high-performance electrocatalysts with minimal precious metal content and cost (1–16). Specifically, alloying Pt with non-noble metals can reduce the Pt content of electrocatalysts by increasing their intrinsic activity (1–13). We demonstrated that the formation of a nanosegregated Pt(111)-skin structure over a bulk single-crystal alloy with Pt₃Ni composition enhanced the ORR activity by two orders of magnitude (versus Pt/C catalysts) through altered electronic structure of Pt surface atoms (1). Although these materials cannot be easily integrated into electrochemical devices, their outstanding catalytic performance needs to be mimicked in nanoparticulate materials that offer high surface areas. Caged, hollow, or porous nanoparticles offer a promising approach for meeting these performance goals. The hollow interior diminishes the number of buried nonfunctional precious metal atoms, and their uncommon geometry provides a pathway for tailoring physical and chemical properties. They have thus attracted increasing interest in fields such as catalysis, biomedical materials, and electronics (5–7, 19–27).

Hollow nanostructures have been prepared by template-directed protocol relying on the removal of microbeads or nanobeads, treatments based on the Kirkendall effect and the galvanic displacement reaction (19–28). Here, we present a novel class of

electrocatalysts that exploit structural evolution of bimetallic nanoparticles; specifically, PtNi₃ solid polyhedra are transformed into hollow Pt₃Ni nanoframes with surfaces that have three-dimensional (3D) molecular accessibility. Controlled thermal treatment of the resulting nanoframes forms the desired Pt-skin surface structure (1, 9). Synthesis of Pt₃Ni nanoframes can be readily scaled up to produce high-performance electrocatalysts at gram scale, and our protocol can be generalized toward the design of other multimetallic nanoframe systems.

We synthesized PtNi₃ polyhedra in oleylamine that had a uniform rhombic dodecahedron morphology and size (20.1 ± 1.9 nm), as observed along three representative zone axes (Fig. 1A, Fig. 2A, and figs. S1 and S2). The oleylamine-capped PtNi₃ polyhedra were dispersed in non-polar solvents such as hexane and chloroform and kept under ambient conditions for 2 weeks, during which they transformed into Pt₃Ni nanoframes (fig. S3) with unchanged symmetry and size (Fig. 1, Fig. 2, and fig. S4). Increasing the solution temperature to 120°C decreased the time needed for this morphological evolution to 12 hours (fig. S5). These conditions were used to trace the entire structural and compositional evolution process at 2-hour time intervals (fig. S6). Samples at three representative stages (0, 6, and 12 hours) were examined by transmission electron microscopy (TEM) (Fig. 1, A to C). The initially solid nanostructures gradually eroded into hollow frames, and the bulk composition changed from PtNi₃ to PtNi and eventually Pt₃Ni, as evidenced by x-ray diffraction (XRD) patterns and energy-dispersive x-ray (EDX) spectra (fig. S7): All three samples are face-centered cubic (fcc), and the three main XRD peaks for each sample—(111), (200), and (220)—are located between those for Pt and Ni; during the evolution process, the peaks shifted toward lower angle (increased *d* spacing), which suggests that the nanostructures had changed from Ni-rich to Pt-rich alloys, in accordance with the EDX results. After dispersion of nanoframes onto a carbon support with high surface area (Vulcan

XC-72) and subsequent thermal treatment in inert gas (Ar) atmosphere between 370° and 400°C, most nanoframes developed the smooth Pt-skin type of structure (Fig. 1D).

High-resolution TEM (HRTEM) showed that the initial PtNi₃ polyhedra were fcc nanocrystals (Fig. 2). For the hollow Pt₃Ni nanoframes, high-angle annular dark-field scanning TEM images showed an architecture consisting of 24 edges (width ~2 nm) of the parent rhombic dodecahedron (Fig. 2B) that maintained the single-crystalline structure (figs. S2 and S8).

In contrast to other synthesis procedures for hollow nanostructures that involve corrosion induced by harsh oxidizing agents or applied potential, the method described here proceeds spontaneously in air through free corrosion. We followed the compositional evolution of these framed, bimetallic nanostructures with x-ray photoelectron spectroscopy (XPS). In the presence of dissolved oxygen, the surface Ni atoms are more susceptible to oxidation than Pt atoms. The Ni 2p and Pt 4f XPS spectra of PtNi₃ polyhedra obtained in vacuum (Al K α , *h* ν = 1486.6 eV) show that the majority of the surface Ni was oxidized and the surface Pt was mainly in the metallic state (Fig. 2, D and E). Oxidized Ni can readily form soluble metal complexes with the oleylamine ligands (29) and lead to a higher dissolution rate for Ni versus Pt that drives compositional change from Ni-rich to Pt-rich, until the stable Pt₃Ni phase (30) is formed. The intensity of Pt²⁺ with respect to Pt was barely altered after the system evolved into the final stage (Fig. 2, D and E), whereas the ratio of Ni³⁺ at the surface decreased substantially, implying that oxidation of Ni on the surface became more difficult in the stable Pt₃Ni composition. Additionally, we carried out in situ ambient-pressure XPS studies to examine the changes in surface chemistry of both PtNi₃ and Pt₃Ni in response to the different exposure atmospheres (31), the results of which support the mechanism proposed above (see fig. S10).

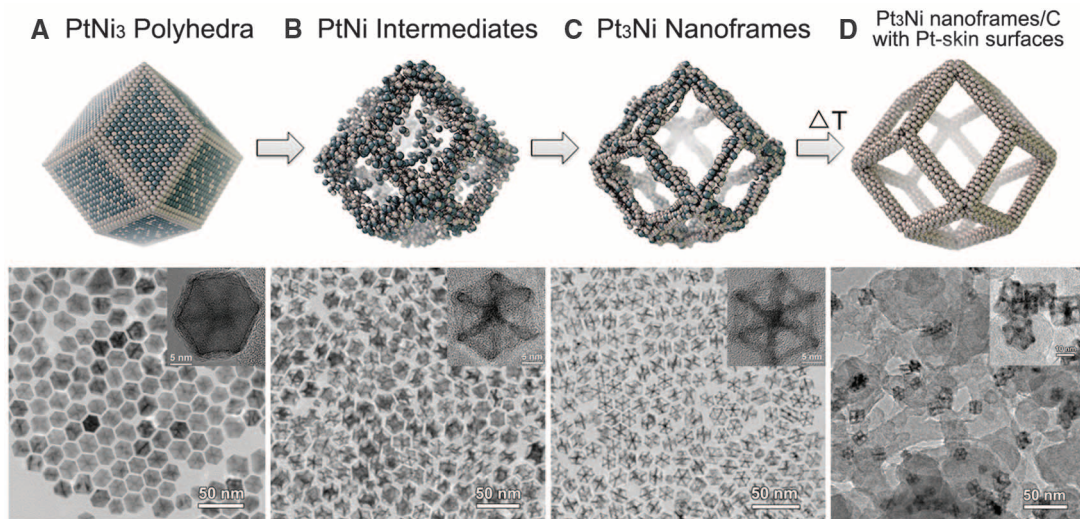
The corresponding morphological changes of the solid polyhedral particles occurred through

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Fig. 1. Schematic illustrations and corresponding TEM images of the samples obtained at four representative stages during the evolution process from polyhedra to nanoframes. (A) Initial solid PtNi₃ polyhedra. (B) PtNi intermediates. (C) Final hollow Pt₃Ni nanoframes. (D) Annealed Pt₃Ni nanoframes with Pt(111)-skin-like surfaces dispersed on high-surface area carbon.



preferential dissolution in the interior of the polyhedral faces, rather than on the edges, driven by an inhomogeneous elemental distribution in the initial nanostructure revealed by TEM (Figs. 1 and 2). The contour of frames could be imaged immediately after synthesis because of the higher Pt content on the edges. EDX elemental mapping (Fig. 2C) and site-specific EDX analyses (fig. S11) for the PtNi₃ polyhedra showed that Ni exhibited a relatively homogeneous distribution inside of the particles, whereas Pt was relatively concentrated at the edges. Such elemental distribution in the original solid polyhedra could be caused by preferential etching of low-coordinated Ni along the edges because of the presence of trace oxygen in the solution during the initial nano-

particle synthesis. During the evolution process to form the nanoframes, this led to stable Pt₃Ni composition on the edges (Fig. 2C), as both Pt and Ni species were removed from the interior of the polyhedra (i.e., as Ni atoms were dissolved, Pt atoms were exfoliated from the Ni-rich interior). Together, the inhomogeneous distribution of Pt on the edges versus the interior and the high dissolution rate of Ni create hollow Pt₃Ni nanoframes containing 24 2-nm-thick edges that retain the high crystallinity of the parent structure (fig. S8).

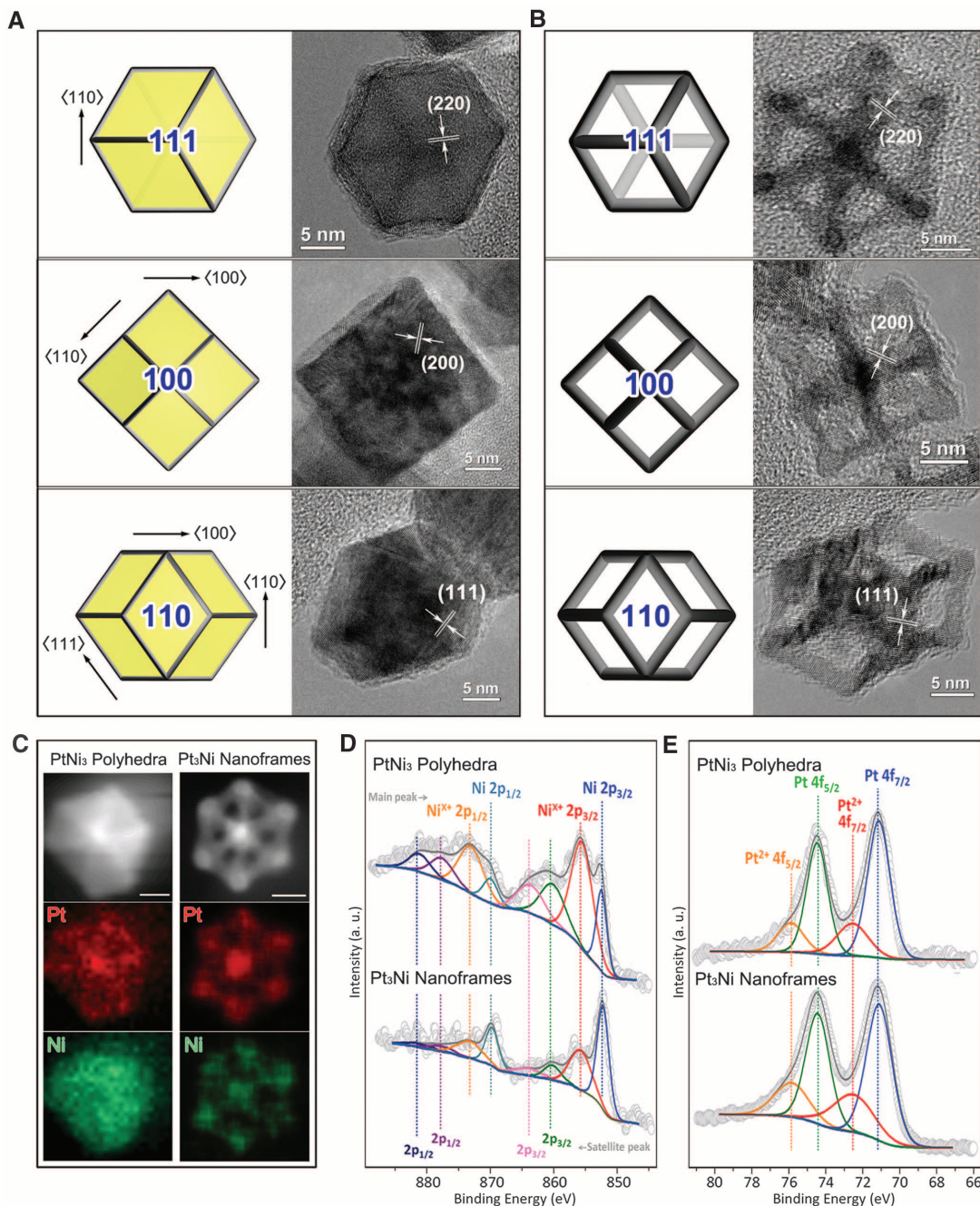
The electrocatalytic properties of Pt₃Ni nanoframes were evaluated and compared to PtNi/C and commercial state-of-the-art Pt/C nanoscale electrocatalysts (Fig. 3). The polarization curves in Fig. 3B show an increase in ORR activity in the

following order: Pt/C < PtNi/C << Pt₃Ni nanoframes. As seen in the Tafel plot (Fig. 3C), Pt₃Ni nanoframes exhibited substantially higher activity, with a slope of 46 mV dec⁻¹ (versus 73 mV dec⁻¹ for Pt/C), which is in agreement with that of Pt₃Ni (111)-Pt-skin (3). The kinetic current densities representing the intrinsic activities were calculated by the Koutecky-Levich equation and summarized in Fig. 3, E and F, as specific and mass activities, respectively. The specific activities were calculated through normalization by the electrochemically active surface area (ECSA) as estimated by CO stripping (electro-oxidation of adsorbed CO).

The ratio between ECSA values determined by integrated charge from CO stripping (ECSA_{CO}) and underpotentially deposited hydrogen (ECSA_{Hupd})

Fig. 2. Characterization of the initial PtNi₃ polyhedron and final Pt₃Ni nanoframes.

(A) Three typical principle projections of the initial PtNi₃ polyhedron revealed a morphology of solid rhombic dodecahedron with single crystallinity. (B) The final Pt₃Ni nanoframe well inherits the symmetry and single crystallinity of parent PtNi₃ polyhedron, with a hollow interior developed and 24 edges (width ~2 nm) remaining. For each projection of both initial polyhedron and final nanoframe, the schematic illustration (left) and HRTEM image (right) are shown. (C) EDX elemental mapping results for PtNi₃ polyhedron and Pt₃Ni nanoframe, suggesting that Ni is homogeneously distributed whereas Pt in the parent PtNi₃ has a slightly higher ratio on the edges (scale bar, 5 nm). (D) Ni 2p and (E) Pt 4f XPS spectra of PtNi₃ polyhedra and Pt₃Ni nanoframes, from which it can be seen that, during the evolution process, the intensity of Ni²⁺ relative to Ni decreases whereas the relative ratio of Pt²⁺ to Pt is barely changed.



was 1.52 for the Pt₃Ni nanoframes, which strongly suggests the formation of a Pt-skin-terminated (111)-like surface structure (Fig. 3A and table S1) (4). Moreover, EDX line profiles confirmed the presence of Pt-skin on the nanoframe surfaces with a thickness of at least two Pt monolayers (MLs) (fig. S13). As a result, the specific activity of Pt₃Ni nanoframes at 0.95 V exhibited an improvement factor of >16 versus commercial Pt/C electrocatalyst (Fig. 3E). The extraordinarily high activity of the Pt₃Ni nanoframes, combined with the distinct ECSA_{CO}/ECSA_{H_{upd}} ratio and EDX line profile, is indicative of a Pt₃Ni–Pt-skin formation, with a topmost Pt-skin thickness of at least 2 MLs rather than 1 ML, which is common for ideal bulk Pt₃Ni(111) single crystal. Despite that divergence, 2 MLs of Pt-skin indeed sustain the enhancement in the ORR rate that is based on altered electronic structure of Pt topmost atoms by subsurface Ni, causing a lower surface coverage of spectator oxygenated species, namely OH_{ad}, and hence superior catalytic properties (1).

Surface strain of the Pt atoms also contributes to the functional properties of the nanoframes. The influence of strain on catalytic behavior was evaluated by density functional theory (DFT) simulations in which dependence of activity versus Pt-skin thickness was estimated to be optimal for 2 to 3 MLs (see fig. S15). These findings shed light on the origin of the high catalytic activity as well as the durability of the catalyst. The Pt-skin surface structure of the nanoframes in conjunction with their high ECSA provides the link between well-defined extended surfaces and highly crystalline nanoscale electrocatalysts. The synergy between specific activity and the open architecture of the Pt₃Ni nanoframes that enables access of reactants to both the internal and external surfaces (fig. S16) led to a factor of 22 enhancement in mass activity versus Pt/C catalysts (Fig. 3F). The mass activity calculated at 0.9 V (5.7 A mg^{−1} Pt) is more than an order of magnitude greater than the U.S. Department of Energy's 2017 target (0.44 A mg^{−1} Pt).

In addition to the high intrinsic and mass activities, the Pt₃Ni nanoframes exhibited remarkable durability throughout electrochemical operation. We cycled the potential between 0.6 and 1.0 V for a duration of 10,000 potential cycles at different sweep rates from 2 to 200 mV s^{−1}. For the state-of-the-art Pt/C electrocatalysts, such cycles cause substantial loss of specific surface area (~40%) because of dissolution of Pt surface atoms and agglomeration of Pt particles through surface oxidation/reduction processes (8, 18). In contrast, STEM (dark-field and bright-field) images confirmed that the frame structure was preserved while activity loss was negligible after 10,000 potential cycles (Fig. 4). The enhanced durability is ascribed to the electronic structure of the Pt-skin surface resulting in a lower coverage of oxygenated intermediates because of the weaker oxygen binding strength, which diminishes the probability of Pt dissolution (1). In addition, the optimized Pt-skin thickness of at least 2 MLs hinders the loss of subsurface transition metal

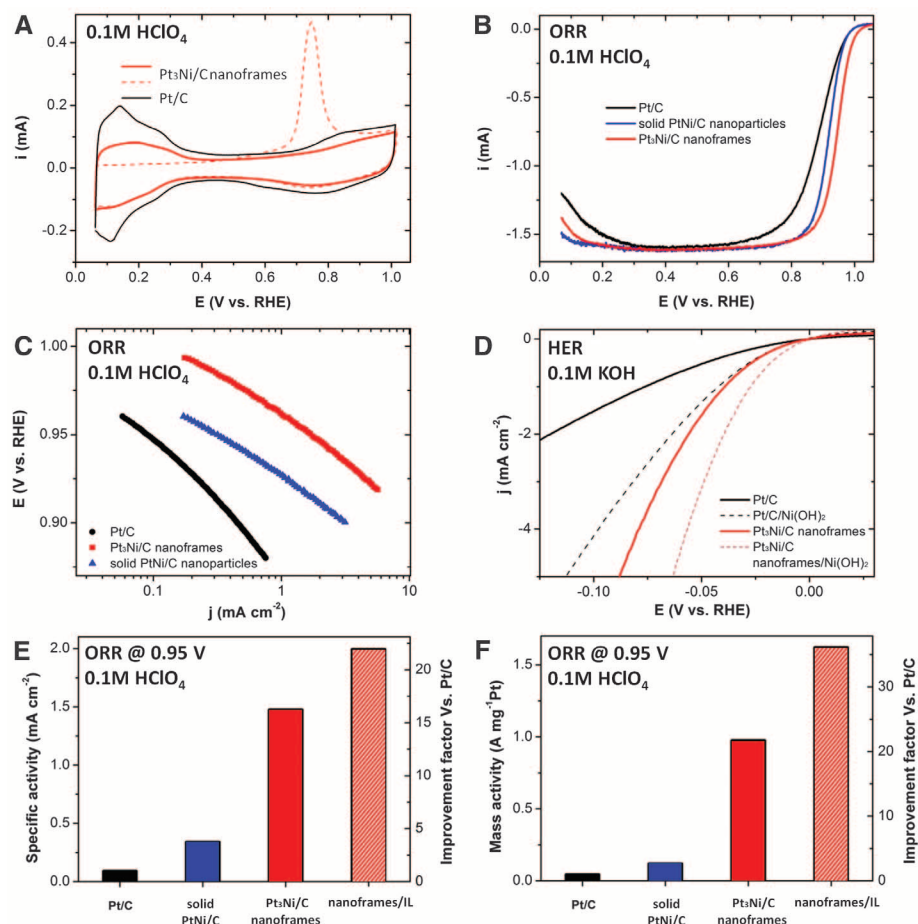


Fig. 3. Electrochemical properties of Pt₃Ni nanoframes. (A) Cyclic voltammograms of Pt/C and Pt₃Ni/C nanoframes signify the difference in surface coverage by H_{upd} and OH_{ad}. ECSA of the nanoframes is determined by integrated charge of adsorbed CO electro-oxidation curve. (B) ORR polarization curves. (C) The corresponding Tafel plots. (D) HER activities for Pt/C, Pt₃Ni(OH)₂/C, Pt₃Ni nanoframes/C, and Pt₃Ni frames/Ni(OH)₂/C in alkaline electrolyte. (E and F) Specific activities (E) and mass activities (F) measured at 0.95 V, and improvement factors versus Pt/C catalysts. Because of the high intrinsic activity of the Pt₃Ni nanoframes, the ORR activity values are given at 0.95 V in order to avoid the extensive error margin at 0.9 V introduced by the close proximity of current values to the diffusion-limited current. IL, ionic liquid.

through the place-exchange mechanism during electrochemical operation, consequently preserving the high intrinsic activity (9). The Pt₃Ni nanoframe structure was retained after annealing at 400°C in Ar for several hours, demonstrating its thermal stability (fig. S19).

As reported by Erlebacher and co-workers, protic ionic liquids can be integrated into a nanoporous catalyst, where the high O₂ solubility of ionic liquids increases the O₂ concentration at the catalyst surface, resulting in higher attempt frequencies for the ORR and consequently higher activity (5, 6). We used [MTBD][NTf₂] (fig. S20), an ionic liquid that has an O₂ solubility (C_{O₂}[MTBD][NTf₂] = 2.28 ± 0.12 mM) approximately twice that of the common electrolyte HClO₄ [C_{O₂}HClO₄ = 1.21 mM (6); see supplementary materials]. Capillary forces exerted by the Pt₃Ni nanoframes pulled the ionic liquid inside the frames and prevented it from being washed away by electrolyte (Fig. 3A and figs. S21 to S23). The ionic

liquid-encapsulated Pt₃Ni nanoframes showed sustained superior activity upon prolonged (10,000) potential cycling without noticeable decay in performance, providing further support for the frame architecture as a desired morphology to fully exploit the beneficial properties of ionic liquids (fig. S24). The ionic liquid-encapsulated Pt₃Ni nanoframes exhibited a factor of 36 enhancement in mass activity and a factor of 22 enhancement in specific activity relative to Pt/C catalysts.

We also applied these electrocatalysts to the HER, which is the crucial cathodic reaction in water-alkali electrolyzers. Electrochemically deposited Ni(OH)₂ clusters on Pt surfaces were recently shown to facilitate dissociation of water, thus increasing the HER activity (14). In the case of highly crystalline Pt₃Ni–Pt-skin nanoframe surfaces modified by electrochemically deposited Ni(OH)₂ clusters (Fig. 3D and fig. S25), the HER activity was enhanced by almost one order of magnitude relative to Pt/C. This result further

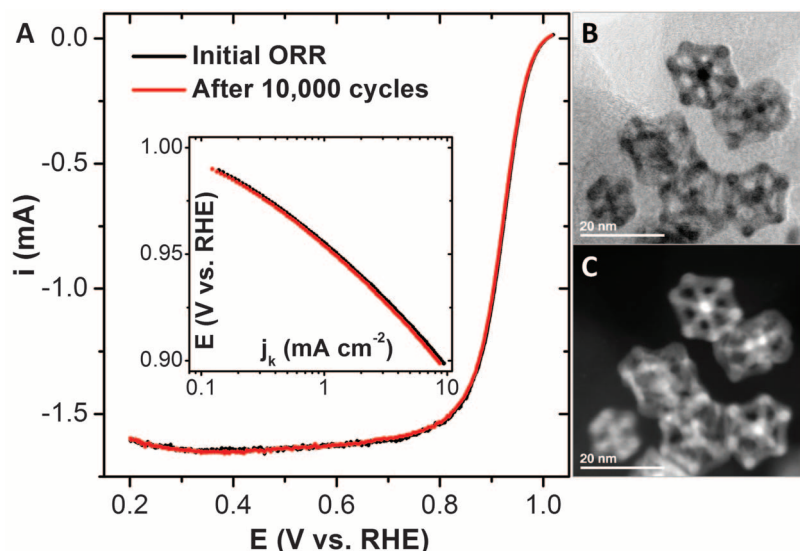


Fig. 4. Electrochemical durability of Pt₃Ni nanoframes. (A) ORR polarization curves and (inset) corresponding Tafel plots of Pt₃Ni frames before and after 10,000 potential cycles between 0.6 and 1.0 V. (B and C) Bright-field STEM image (B) and dark-field STEM image (C) of Pt₃Ni nanoframes/C after cycles.

emphasizes the beneficial effects of the open architecture and surface compositional profile of the Pt₃Ni nanoframes in electrocatalysis.

The open structure of the Pt₃Ni nanoframes addresses some of the major design criteria for advanced nanoscale electrocatalysts, namely, high surface-to-volume ratio, 3D surface molecular accessibility, and optimal use of precious metals. The approach presented here for the structural evolution of a bimetallic nanostructure from solid polyhedra to hollow highly crystalline nanoframes with controlled size, structure, and composition can be readily applied to other multimetallic electrocatalysts such as PtCo, PtCu, PtRh-Ni, and Pt/Pd-Ni (figs. S26 to S29).

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Supplementary Materials

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Materials and Methods
Figs. S1 to S30
Table S1
Movies S1 and S2
References (32–38)

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The Source Crater of Martian Shergottite Meteorites

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Absolute ages for planetary surfaces are often inferred by crater densities and only indirectly constrained by the ages of meteorites. We show that the <5 million-year-old and 55-km-wide Mojave Crater on Mars is the ejection source for the meteorites classified as shergottites. Shergottites and this crater are linked by their coinciding meteorite ejection ages and the crater formation age and by mineralogical constraints. Because Mojave formed on 4.3 billion-year-old terrain, the original crystallization ages of shergottites are old, as inferred by Pb-Pb isotope ratios, and the much-quoted shergottite ages of <600 million years are due to resetting. Thus, the cratering-based age determination method for Mars is now calibrated in situ, and it shifts the absolute age of the oldest terrains on Mars backward by 200 million years.

The martian rock collection contains nearly 150 meteorite specimens: shergottites, nakhlites, and chassignites, as well as one

orthopyroxenite (ALH84001) (1, 2). Currently, the oldest rock linked to Mars appears to be ALH84001, crystallized about 4.1 billion years

ago (Ga) (3, 4) and probably ejected from Mars 20 million years ago (Ma) (5). The original rock unit from which chassignites and nakhlites were ejected about 11 Ma (5) probably crystallized around 1.3 Ga (1, 2, 6). The apparent crystallization ages of shergottites are more diverse and range between 150 and 596 Ma (1, 2, 6, 7). The geochemically depleted [in incompatible elements such as light rare earth elements (LREE) relative to the martian mantle (8)] shergottites cluster at the old age range, whereas the enriched

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and intermediate ones form a group with young ages (1, 2) (fig. S1A). Cosmic ray exposure ages and thus the deduced ejection age for the shergottites occur in clusters between 1 and 5 million years (My) (1, 2, 5), but depletion level and ejection ages appear uncorrelated (fig. S1B).

One indicator of young (<20 Ma) craters is an extended rayed ejecta pattern, and eight such craters have been suggested as meteorite source candidates (9). They range from 2.6 to 29 km in diameter and have been dated by cratering statistics to have formed in the past 20 My (10). We searched for and have identified additional rayed craters (fig. S2). The occurrence of these craters with rayed ejecta patterns is strongly correlated with dusty but old terrains, and none are found on the young volcanoes on the Tharsis plateau. The crater diameters range between 1.5 and 55 km,

and Mojave is the largest crater. The Mojave impact site is centered at 7.5°N and 33.0°W, which is at the outflow channel floor of the Simud and Tiu Valles confluence. The channels were carved during the Hesperian [between about 3.5 and 3.75 Ga, derived by our chronology model (11)], into the Noachian (older than 3.75 Gy) Xanthe Terra plateau, and are about 1500 m deep. Xanthe Terra is one of the oldest units on Mars (12), suggesting that the ejected rocky material originally crystallized in the Early Noachian, or even before. The target has been modified by tectonic and shock-induced brecciation, as well as by aqueous alteration, fluid migration, and volcanic activity, and thus isotope ages determined for such a target can be reset or disturbed by these processes.

The Mojave Crater itself has attracted attention for its well-preserved fluvial landforms

(13–15), which support a very young formation age. However, because of Mojave's large size, age speculations range from Late Hesperian (16) to Early Amazonian (15, 17) (younger than 3.5 Gy) age. Statistically, a 55-km-sized crater could form every 35 to 50 My (18). Mojave Crater exposes features similar to the crater class of very young rayed craters (Fig. 1A), and chains of secondary crater clusters coinciding with ray patterns observed on THEMIS (Thermal Emission Imaging System on the Mars Odyssey spacecraft) nighttime mosaics are present on the plateau units and on the floors of Simud and Tiu Valles at distances as far as 1000 km away (Fig. 1B). Mojave's near-field secondary craters reach a maximum size of less than 1 km in diameter (normally 2 to 5% of primary crater diameter), suggesting a strong brecciation of the target rock.

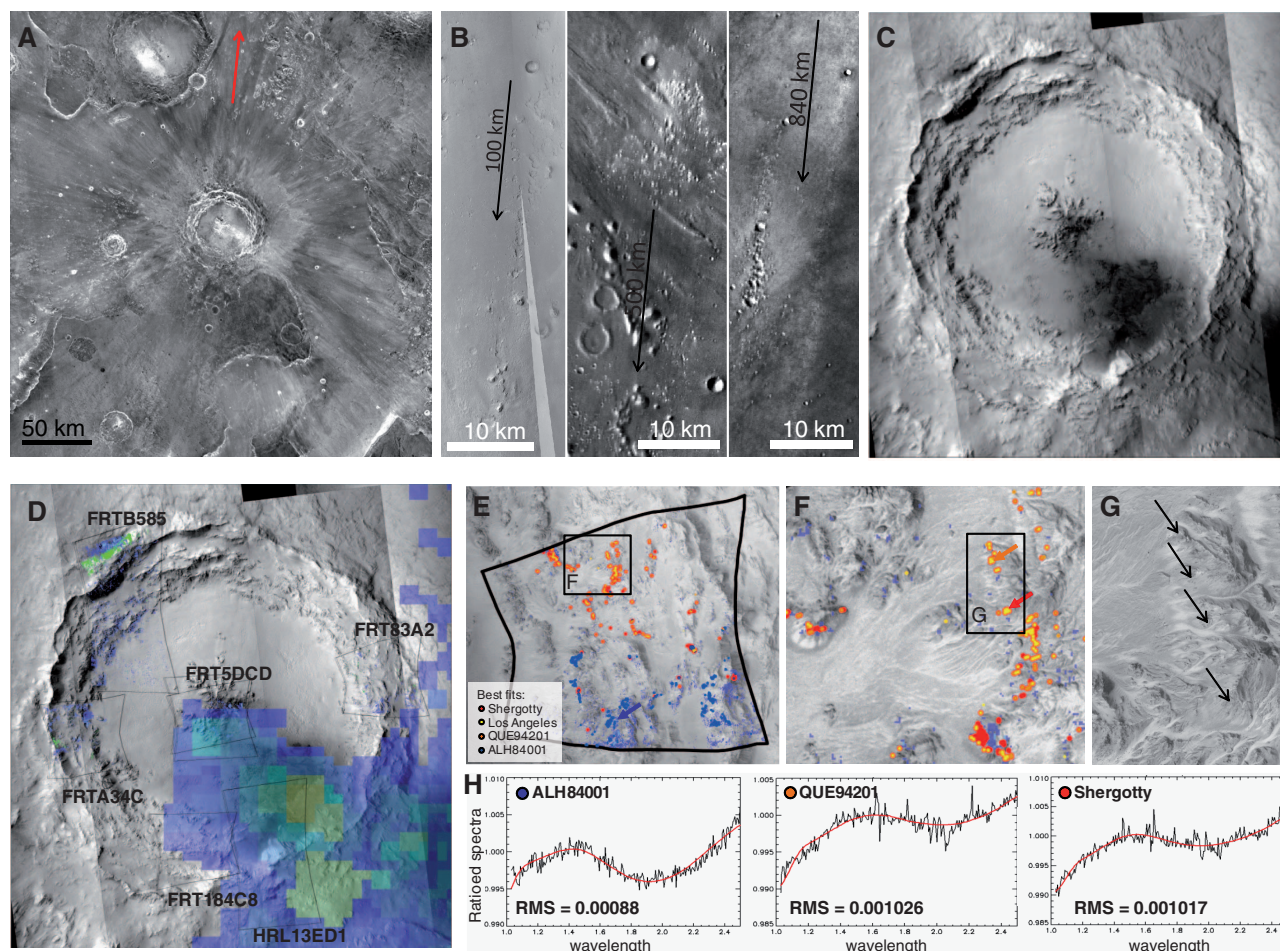


Fig. 1. Morphology and mineralogy of Mojave Crater. (A) THEMIS nighttime image of Mojave Crater showing its well-preserved morphology and ray pattern. The direction of the red arrow indicates distant examples of secondary crater clusters shown in (B). (B) Examples of Mojave's secondary crater clusters at 100, 500, and 840 km distance along the same ray indicated in (A) (red arrow). (C) Mars Reconnaissance Orbiter Context Camera image mosaic of Mojave Crater's interior (D) overlain by OMEGA pyroxene detections (color range blue to green, large pixels) and CRISM pyroxene (blue, small pixels) and olivine (green, small pixels) detections of the Mojave Crater region. The footprints and names of CRISM observations are indicated. (E) Locations of best fits obtained by fitting the four martian meteorite spectra and spectra of

the CRISM FRT83A2 observation (D) of the eastern wall of Mojave Crater are plotted together with the pyroxene detection map. Best fits are indicated with red points for Shergotty, orange points for QUE94201, yellow points for Los Angeles, and blue points for ALH84001. Close-ups of this map (F and G) show that the best fits for shergottites are associated with rocky outcrops at the hill flanks indicated by arrows on (G). A similar geological context is found for ALH84001 in the southern part of the CRISM observation (E). (H) Examples of fits for the ALH84001, Shergotty, and QUE94201 martian meteorites extracted from locations marked by colored arrows in (E) and (F). The processed CRISM spectra are shown as black curves and the fitted meteorite spectra as red curves.

Numerical models (19) suggest that martian meteorite material was launched from an annular area of 2.5 to 5 projectile radii distance from the impact site center and at very shallow depths of less than 0.2 projectile radius (i.e., ~500 m). Given their shallow depth, materials detected in the final crater setting may best represent the characteristics of the ejected material.

Using near-infrared hyperspectral data from OMEGA (Observatoire pour la Minéralogie, l'Eau, les Glaces et l'Activité, European Space Agency Mars Express) and CRISM (Compact Reconnaissance Imaging Spectrometer for Mars, NASA Mars Reconnaissance Orbiter), we evaluated Mojave's crater walls, central peak, and adjacent erosional products (debris aprons near outcrops and large-scale dark patches, Fig. 1C) for spectral signatures of pyroxene and olivine, two minerals that are commonly present in the martian meteorites (Fig. 1D). Although all observations are largely disturbed by surface dust and substantial atmospheric aerosol opacity, both minerals are detected in specific terrains: We observed the pyroxene signature in several dust-free outcrops located in crater walls and in the central peak, as well as in sand sheets around the central peak and in the southeastern sector of the crater interior. We detected olivine in fewer occurrences, and it is found mainly in localized outcrops (Fig. 1D).

To better assess the compositional similarity of martian meteorites and the mafic terrains of Mojave Crater, we compared each CRISM and OMEGA pixel exhibiting the pyroxene signature to spectra of pyroxene-bearing martian meteorites: the orthopyroxenite ALH84001, the "enriched" shergottites Shergotty and Los Angeles (20), and the depleted shergottite QUE94201 (21). Then we evaluated the comparison by a root mean square (RMS) fit. For OMEGA spectra, no satisfactory fits [$\text{RMS} < 0.2\%$, see (11)] were obtained. Pyroxene-indicating CRISM spectra (FRT0000A34C, FRT000083A2, and FRT0005DCD) are well reproduced by the meteorite spectra of ALH84001 (which is almost pure orthopyroxenite) and those of enriched and depleted basaltic shergottites, with the most numerous fits found for Shergotty (which is a mixture of pyroxene and shocked plagioclase/maskelynite). The detections are associated with rocky outcrops in the crater walls (both the eastern and western sides) and at the crater peak, as well as in sand sheets of these regions, possibly sourced locally by material eroded from these outcrops (Fig. 1, E to H). Satisfactory fits are also found for central peak materials. These materials are excavated from deeper layers than the meteorite source zone and cannot be directly associated with meteorite material, but support a shergottite-like crustal composition. Based on reflectance spectroscopy (11), the mineralogy of the Mojave Crater wall outcrops matches the composition of ALH84001 (orthopyroxene) and both enriched-intermediate and depleted basaltic shergottites [(clino)pyroxene-plagioclase and minor olivine].

To determine the formation age of Mojave Crater, we performed crater counts on Mojave's entire continuous ejecta blanket and separately on the surrounding channel floors and plateau units (Fig. 2). We excluded the crater interior because of numerous pits resulting from target volatile degassing during crater formation (22) and units obviously disturbed by secondary cratering of Mojave. The evolutionary history of the impact site starts with the formation of the Noachian Xanthe Terra plateau unit crust, and its age is indicated by the largest craters (>50 km in diameter). A subsequent, secondary deposition event, commonly described as intercrater plains volcanism (12), follows (craters between 10 and 50 km in diameter). The largest craters at the channel floor (>3.5 km in diameter) indicate the time at which the channel's incision ended. The channel floors were modified subsequently (<3.5 km in diameter) by a depositional episode, which was either volcanic or fluvial. For the continuous ejecta blanket, the crater density at the large diameter range ($D > 1.1$ km) matches those elsewhere on the channel floor of craters

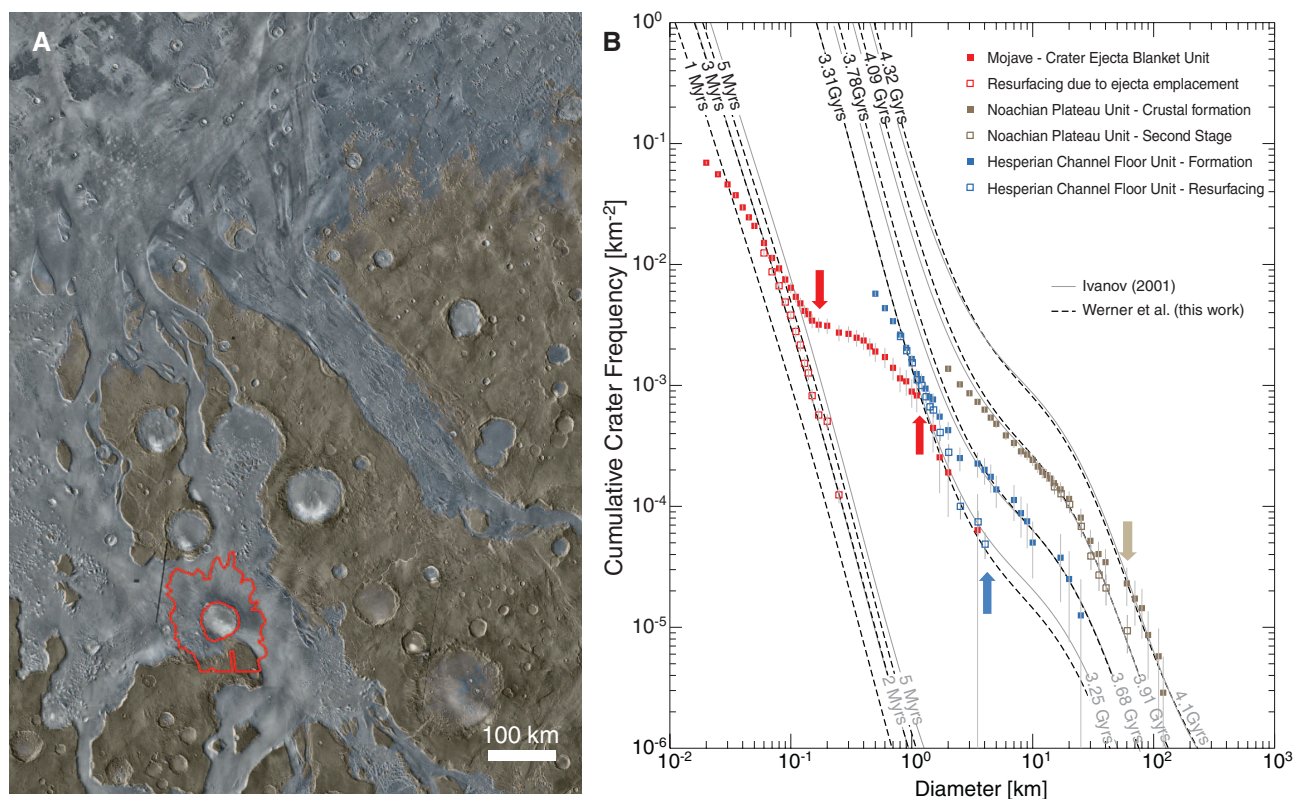


Fig. 2. Crater statistics of Mojave. (A) THEMIS daytime image mosaic overlain by Mars Orbiter Laser Altimeter color-coded topography. Craters were counted for the plateau units (brown), the channel units (blue-gray), and the continuous ejecta unit of Mojave Crater (red line). (B) Results of the crater count statistics [brown, blue-gray, and red refer to the units in (A)], displayed

as cumulative crater frequency versus crater diameter. The age interpretation is based on our crater-production function and cratering chronology model (black dashed line) and is compared to a standard model [(23), light gray solid line]. Colored arrows indicate the onset crater diameter, at which resurfacing occurs or stops.

of comparable size. However, the cumulative crater size-frequency distribution reveals a single, notable resurfacing event (Fig. 2B). This well-pronounced resurfacing event and the removal of craters emerge for diameters between 0.25 and 1.1 km. The shape of the crater size-frequency distribution testifies that this event occurred very quickly, rather than by continuous infill of, for example, aeolian sediments or cyclic deposits under obliquity variations. Using simple crater morphometry rules, both the estimated ejecta blanket thickness of Mojave Crater and the depth-diameter relation of the erased craters in the ejecta units yield an average deposit layer thickness of about 200 to 250 m. The larger craters are less buried at the ejecta blanket edges, which is in agreement with the ejecta thickness thinning with the distance from the crater center. Thus, the sudden and strong emplacement of material must have been caused by the ejecta emplacement itself.

For the determination of the absolute model ages, we developed a martian (and lunar) cratering chronology model (11). This model describes a lower maximum flux and a slower decay rate and results in about 200-My older ages for the same crater densities on Mars. Because martian target materials have higher effective strength (23) than lunar materials, the ratio between large and small craters is therefore larger and is considered for the isochrons. We compared the isochrons for our model with those for one (18) commonly used standard model (Fig. 2B). The craters superposing the ejecta indicate an absolute model age of about 3 Ma for a fit to craters in the diameter range of 50 to 250 m, and thus a formation age for Mojave of about 3 Ma. The observed crater size-frequency distribution for crater diameters smaller than about 50 m significantly differs from the predicted isochron (Fig. 2B), implying resurfacing identified in the images in agreement with continuous aeolian deposition at a maximum rate of 3 m/My. The isochron-derived cumulative crater-production rate for craters larger than and equal to 16 m is $3.6 \times 10^{-7}/\text{km}^2/\text{year}$, whereas the observed crater retention age is only $7.4 \times 10^{-8}/\text{km}^2/\text{year}$. The latter agrees well with today's observed rate (24) ($9 \times 10^{-8}/\text{km}^2/\text{year}$). Using the very low observed cratering rate (25) for craters smaller than 16 m for inferring the age of Mojave, the age could be up to 8 My. A complete understanding relies on appropriate crater-scaling relations for the strength regime, effective material properties, and seasonal cratering rate variations (23). Any of these parameters would increase the absolute model age of Mojave Crater, because the age uncertainty is about a factor of 2.

The formation age of Mojave Crater is as young as 3 Ma, making it the youngest crater of this size (10). It is larger than any other rayed crater on Mars (9, 10), none of which are found on the young volcanoes on the Tharsis plateau. The oldest martian surface units (Xanthe Terra) must be about 4.3 Gy old (200 My older than in previous cratering chronology models), and the intercrater plains volcanism occurred 4.1 Ga. The

old age of the target terrain of Mojave Crater would restrict the Mojave Crater-meteorite connection to the orthopyroxenite ALH84001. The recent impact event at about 3 Ma that formed Mojave, though, better fits the ejection ages of shergottites, which cluster between 1 and 5 My (5). Given the site mineralogy, all shergottites and possibly even ALH84001 must have been ejected by a single impact event that formed Mojave Crater. The variation of 1 to 5 My in cosmic ray exposure ages is interpreted to be the result of breakup events during transit to Earth (5), because older cosmic ray exposure ages can result from previous surface exposure on the planet (5).

To reconcile the apparently young (and often discordant) crystallization ages of shergottites with the very old age of Xanthe Terra, we propose that the original crystallization ages could have been underestimated for shergottites, because of processes such as impact melting or shock-induced resetting (26), as well as aqueous alteration processes (27). The lobate-shaped ejecta blanket, the pitted crater floor, and the fluvial landforms in the crater interior are evidence for substantial amounts of subsurface water (ice) being present at the impact site before and heated during the cratering event. The magnetic carrier pyrrhotite (iron sulphide), is present in many shergottites, basaltic shergottites being magnetic enough to account for the observed crustal remanent magnetization on Mars (28). This carrier is a host for rare earth elements used for dating and is prone to thermal and shock-released heat alteration and can explain the moderate magnetization of the crust surrounding the Mojave impact site.

All martian meteorites have experienced shock metamorphism (26). The launch event requires only about 10 GPa. Such a pressure is sufficient to melt baddeleyite and form new zircons (29), suggesting that the higher pressures recorded by mineral transformation in shergottites, such as plagioclase to maskelynite (26), may have been introduced before the launch event (19). Although their significance is debated, the 4.1 Gy and 4.3 Gy old Pb-Pb ages for ALH84001 and shergottite groups (3, 27, 30, 31) are most consistent with the age of the Xanthe Terra terrain. This terrain has experienced several large enough impacts that produced the observed shock metamorphism. The group of depleted shergottites could thus represent the oldest material known from Mars, with an average age of 4.32 Gy (3, 27, 30, 31), whereas the group of enriched and intermediate shergottites are of the same age as the meteorite ALH84001, and on average 4.09 Gy old (3, 27, 30, 31). We therefore conclude that all of these meteorites were sourced from the Mojave impact site.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S3
Table S1
References (32–51)

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Iron Fertilization of the Subantarctic Ocean During the Last Ice Age

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John H. Martin, who discovered widespread iron limitation of ocean productivity, proposed that dust-borne iron fertilization of Southern Ocean phytoplankton caused the ice age reduction in atmospheric carbon dioxide (CO₂). In a sediment core from the Subantarctic Atlantic, we measured foraminifera-bound nitrogen isotopes to reconstruct ice age nitrate consumption, burial fluxes of iron, and proxies for productivity. Peak glacial times and millennial cold events are characterized by increases in dust flux, productivity, and the degree of nitrate consumption; this combination is uniquely consistent with Subantarctic iron fertilization. The associated strengthening of the Southern Ocean's biological pump can explain the lowering of CO₂ at the transition from mid-climate states to full ice age conditions as well as the millennial-scale CO₂ oscillations.

Large expanses of the open ocean surface are characterized by high concentrations of the inorganic nutrients nitrate and phosphate, which are expected to—but do not—lead to highly productive conditions. These “high-nutrient, low-chlorophyll” (HNLC) regions stood as one of the great mysteries of oceanography until the late John H. Martin and colleagues reported evidence that phytoplankton growth in HNLC regions is limited by iron (1, 2). Since that time, numerous lab and field studies, including campaigns of open ocean iron addition, have confirmed that iron availability is central to the dynamics of HNLC regions (3). However, even the most ambitious open ocean experiments have not yielded a predictive understanding of how the productivity in (and carbon export from) HNLC regions would respond to natural, large-scale, long-term changes in iron supply.

Carbon dioxide has varied in step with climate over at least the past 800,000 years (4), and it is broadly suspected to play a central role in translating the Milankovitch cycles in Earth's orbital parameters into the glacial-interglacial climate cycles. Soon after the discovery of glacial-interglacial CO₂ changes, the Southern Ocean was recognized as a potential driver. The modern Southern Ocean releases deeply sequestered CO₂ to the atmosphere, leading to the hypothesis that the Southern Ocean CO₂ “leak” was stemmed during ice ages, thereby increasing ocean CO₂ storage (5–7). Recognizing the convergence of these ideas with his evidence for iron limitation of phytoplankton in the modern Southern Ocean (2) and Antarctic ice core evidence of greater

deposition of iron-rich dust during ice ages (8), Martin proposed the “iron hypothesis”: that iron fertilization of the ice age Southern Ocean caused an increase in productivity, which increased the rain of organic carbon into isolated deep waters (“export production”) and thus contributed to the reduction in atmospheric CO₂ observed during ice ages (9). Subsequent sediment reconstructions indicated that ice age productivity was higher in the Subantarctic Zone, the more northern domain of the Southern Ocean, but lower in the Antarctic Zone, the Southern Ocean region closest to the Antarctic continent. This heterogeneous productivity change caused the iron hypothesis to lose favor, with the glacial productivity variations typically interpreted as the result of a northward shift in Southern Ocean fronts (10).

More recently, Southern Ocean hypotheses for glacial-interglacial CO₂ change, including the iron hypothesis, have been revisited. It has been proposed that the Antarctic Zone was more strongly stratified during ice ages, leading to less exposure of nutrient- and CO₂-rich deep waters at the Antarctic surface as well as more complete consumption of the reduced supply of upwelled nutrients (11). This process is consistent with the observation of lower glacial marine export production and can explain up to ~40 parts per mil-

lion (ppm) of the ice age CO₂ decline (12, 13). In the Subantarctic, a strong correlation between proxies of aeolian iron flux and productivity has rekindled the iron hypothesis in its pure form (14, 15), which is particularly compelling for this region because it sits downstream of the ice age continental dust plumes (Fig. 1) (16). The increases in Subantarctic iron flux and productivity are timed appropriately to explain the latter ~40 ppm of the CO₂ decline that occurred over the last glacial cycle (15, 17, 18).

Given the observed coupling of cooling, higher dust flux, and increased Subantarctic productivity, a key remaining test for the iron hypothesis in the Subantarctic is whether surface nutrient concentrations declined. If the ice age increase in Subantarctic productivity was due to iron fertilization, then the “major” nutrients (nitrate and phosphate) in surface waters would have been more completely consumed in the production of phytoplankton biomass. This increase in the degree of consumption would have worked to lower their concentrations in the Subantarctic surface. In contrast, if the ice age increases in Subantarctic productivity resulted from equatorward migration of productive Southern Ocean fronts, then nitrate and phosphate concentrations would have increased in the region during the ice ages.

Changes in the degree of nitrate consumption by phytoplankton can be reconstructed using the isotopes of nitrogen. Isotope fractionation during the assimilation of nitrate by phytoplankton leads to a $\delta^{15}\text{N}$ increase in Subantarctic surface nitrate and biomass N as nitrate consumption proceeds, where $\delta^{15}\text{N} = [(^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{air}}] - 1$ (19–21). The first attempts to reconstruct surface nutrient conditions during ice ages in this manner were based on analyses of bulk sediment samples (11, 22). During the Last Glacial Maximum (LGM, 26,500 to 19,000 years ago), bulk sediment $\delta^{15}\text{N}$ was found to be lower in the Subantarctic Atlantic, the sector of the Subantarctic that hosted the most distinct productivity increase during the LGM (11). This result taken at face value would suggest that the Subantarctic increase in export production was

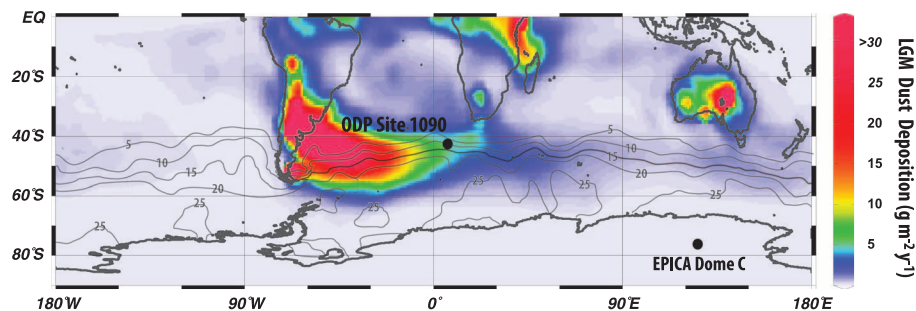


Fig. 1. ODP Site 1090 relative to modern nitrate concentration and ice age dust deposition. Black contours indicate climatological surface nitrate concentration (micromolar values) during austral summer, December to February (40). Colors reflect model-reconstructed dust deposition ($\text{g m}^{-2} \text{ year}^{-1}$) during the LGM (16). The location of the European Project for Ice Coring in Antarctica (EPICA) Dome C is also indicated.

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associated with an even greater increase in nitrate supply and thus a decline in the degree of nitrate consumption, arguing against the iron fertilization hypothesis and in support of northward migration of the Southern Ocean fronts. However, potential biases associated with bulk sediment $\delta^{15}\text{N}$, including diagenetic alteration (18) and potential contamination by terrestrial and shelf nitrogen (23), rendered this finding uncertain.

Recent efforts to overcome the concerns of organic matter alteration and contamination have focused on the analysis of organic N bound within diatom microfossils [diatom-bound $\delta^{15}\text{N}$ (DB- $\delta^{15}\text{N}$)]. In contrast to the bulk sediment reconstructions, DB- $\delta^{15}\text{N}$ records indicated a rise in $\delta^{15}\text{N}$ in all sectors of the Subantarctic, supporting the iron fertilization paradigm (24, 25). However, the DB- $\delta^{15}\text{N}$ records are from cores in the more polar Subantarctic, where silicate availability is adequate to yield significant diatom opal in the sediment. As a result, the records may be complicated by migration of the fronts between the Antarctic and Subantarctic, as suggested by complex opal-associated productivity changes (26). Moreover, the DB- $\delta^{15}\text{N}$ measurements included the entire diatom assemblage, leading to uncertainties associated with the isotopic impact of glacial-interglacial diatom species changes (27). Finally, in this region of currently deep mixed layers, physiologically driven changes in the amplitude of isotope fractionation associated with nitrate assimilation by diatoms may have occurred in response to ice age conditions, yielding DB- $\delta^{15}\text{N}$ changes independent of nitrate consumption (25, 28, 29).

Two decades after the first attempts (30), analytical advances now allow for the isotopic analysis of the organic N bound within the calcium carbonate shells of planktonic foraminifera [foraminifera-bound $\delta^{15}\text{N}$ (FB- $\delta^{15}\text{N}$)] (23, 31, 32). Unlike DB- $\delta^{15}\text{N}$, FB- $\delta^{15}\text{N}$ can be applied in silicate-poor oceanic regions such as the Subantarctic Zone well to the north of the Antarctic and Polar Frontal Zones. FB- $\delta^{15}\text{N}$ has been shown to covary with the $\delta^{15}\text{N}$ of the nitrate consumed in surface waters (31), which increases with the degree of nitrate consumption in nutrient-rich regions such as the Southern Ocean. The N isotope dynamics associated with nitrate consumption are propagated to foraminifera by their feeding on phytoplankton and/or on zooplankton or detritus generated from phytoplankton biomass (23, 31, 32). The $\delta^{15}\text{N}$ relationship between the nitrate consumed in surface waters and foraminifera-bound N varies among foraminifera species (31), but individual species can be picked for isotopic analysis.

Here, we present a record of FB- $\delta^{15}\text{N}$ from the Subantarctic Zone, in the sediment core from Ocean Drilling Program (ODP) Site 1090 in the eastern South Atlantic (Fig. 1). Today, this site is characterized by high unused concentrations of nitrate, but it is located in the downwind plume of the Patagonian dust source that is believed to dominate ice age Southern Ocean dust supply

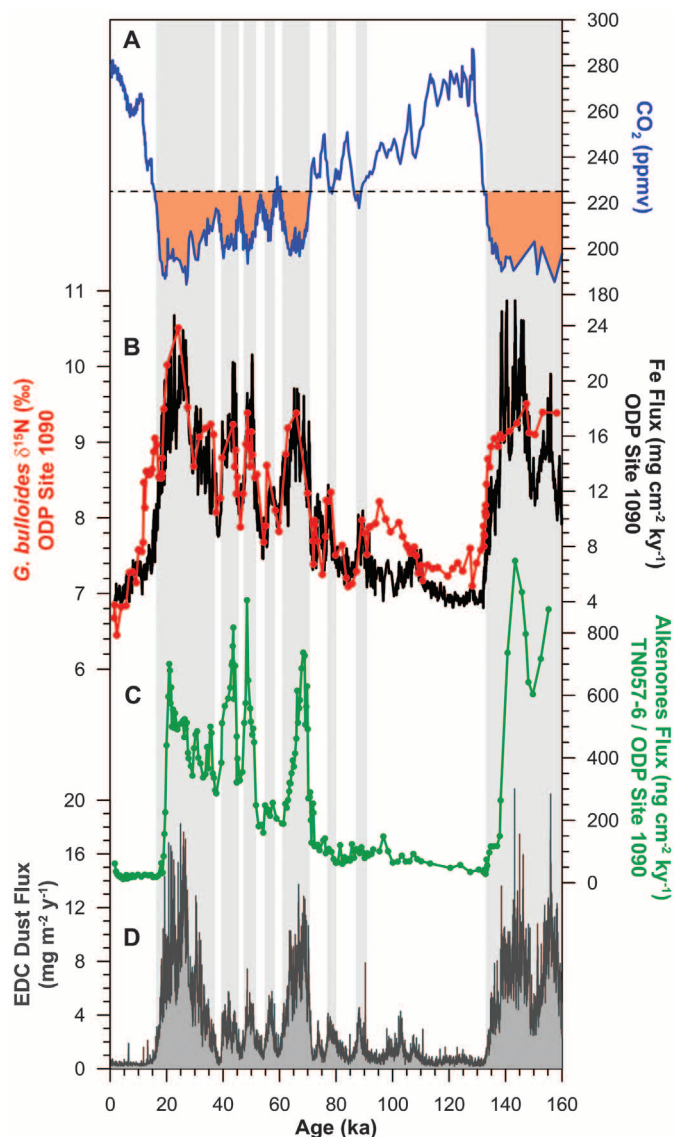
(16) (Fig. 1). This makes the core ideal to test for the effect of increased iron deposition on export production and major nutrient consumption during the last ice age. The FB- $\delta^{15}\text{N}$ record was generated using the spinose, asymbiotic species *Globigerina bulloides*. These data were combined with a high-resolution, ^{230}Th -normalized reconstruction of dust-borne iron supply and alkenone-based export production (complemented by other productivity proxies in much of the record; see fig. S4). The good agreement among the fluxes of alkenones (a proxy for prymnesiophyte productivity), opal (a proxy for diatom productivity), and bulk nitrogen (which is related to total organic carbon flux) indicates that the observed productivity changes are not limited to a specific phytoplankton group (fig. S4). This suite of measurements allows us to directly compare variations in dust deposition, marine export production, and nutrient consumption in the same sediment record.

FB- $\delta^{15}\text{N}$ is higher during both the last ice age and the previous ice age when iron and alke-

none fluxes are elevated, as the iron hypothesis would predict (Fig. 2). Iron fertilization would allow phytoplankton to more completely consume the major nutrients. The more complete consumption of nitrate would cause a rise in the $\delta^{15}\text{N}$ of the residual nitrate and biomass N, which FB- $\delta^{15}\text{N}$ does indeed record. In contrast, had the ice age increase in Subantarctic productivity been driven by the equatorward migration of productive Southern Ocean fronts, then nitrate concentration at ODP Site 1090 would have risen and the $\delta^{15}\text{N}$ of nitrate and biomass would have fallen, in clear disagreement with the FB- $\delta^{15}\text{N}$ data.

Under the assumption of no change in the concentration of the nitrate supplied to the Subantarctic surface ocean, the FB- $\delta^{15}\text{N}$ constraint on the degree of nitrate consumption can be converted to a surface nitrate concentration by the end of the summer growth period (29). The FB- $\delta^{15}\text{N}$ increase from ~ 6.7 per mil (‰) in the current interglacial to ~ 9.5 ‰ suggests a decline in nitrate concentration from the modern

Fig. 2. Records of Subantarctic dust-borne iron flux, phytoplankton productivity, surface nitrate consumption, and atmospheric CO_2 over the last glacial cycle. (A) Atmospheric CO_2 concentrations measured in Antarctic ice cores (38, 41, 42). The orange shaded area highlights the decline in atmospheric CO_2 that correlates with large dust flux, productivity, and nutrient consumption increases. **(B)** *G. bulloides* FB- $\delta^{15}\text{N}$ (red circles) and ^{230}Th -normalized iron flux from ODP Site 1090 (black line), calculated using the ^{230}Th -normalized mass flux measured in the parallel core TN057-6 (29). **(C)** ^{230}Th -normalized alkenone flux from TN057-6 [0 to 90,000 years ago (90 ka)] and ODP Site 1090 (90 to 160 ka). TN057-6 alkenone concentrations are from (43). **(D)** Dust flux at Antarctic ice core EPICA Dome C (EDC) (44). The gray shaded vertical bars highlight maxima in dust flux that correspond to minima in atmospheric CO_2 .



summer value of $\sim 9 \mu\text{M}$ to $\sim 2 \mu\text{M}$ during much of the last and previous ice ages, and the $\text{FB-}\delta^{15}\text{N}$ of 10.5‰ during the LGM suggests a summer-time nitrate concentration below $1 \mu\text{M}$ (fig. S6). In the context of this calculation, other possible mechanisms to raise the $\text{FB-}\delta^{15}\text{N}$ to the ice age observations include (i) a $\sim 3\%$ increase in the $\delta^{15}\text{N}$ of the nitrate supply, (ii) a $\sim 4\%$ decrease in the isotope effect of nitrate assimilation in this region, and (iii) a $\sim 3\%$ increase in the $\delta^{15}\text{N}$ difference between foraminifera-bound N and the nitrate consumed by phytoplankton (29). The possibility of a change in the nitrate supply is discussed below. A large isotope effect decrease is implausible (29) (fig. S7), and an increase in the $\delta^{15}\text{N}$ difference between *G. bulloides* and assimilated nitrate is not supported by comparison with $\text{FB-}\delta^{15}\text{N}$ in another species, *Orbulina universa* (fig. S5).

Bulk sediment $\delta^{15}\text{N}$ measured in the same intervals does not show a clear glacial-interglacial change (fig. S9), in line with earlier data from the Subantarctic (26). A previous comparison of Subantarctic diatom-bound and bulk sediment $\delta^{15}\text{N}$ suggested that down-core changes in diagenetic

alteration compromise bulk sedimentary N as a recorder of Subantarctic biomass $\delta^{15}\text{N}$ changes (24). This interpretation is strongly supported at Site 1090 by the correlation of the $\delta^{15}\text{N}$ difference between foraminifera-bound and bulk sedimentary N with sedimentary N flux (29) (fig. S9).

The timing of the major $\text{FB-}\delta^{15}\text{N}$ changes follows the expectations of a dominant iron fertilization effect. $\text{FB-}\delta^{15}\text{N}$ increases only weakly from the penultimate peak interglacial [marine isotope stage (MIS) 5e, $\sim 123,000$ years ago] to the first major Antarctic cooling step, $\sim 115,000$ years ago. Instead, the sharp $\text{FB-}\delta^{15}\text{N}$ rise occurs at the transition to MIS 4 ($\sim 70,000$ years ago), when dust flux to Antarctica and the Subantarctic Atlantic underwent its first major rise into the last ice age and when biogenic fluxes rose sharply (Fig. 2 and fig. S4). $\text{FB-}\delta^{15}\text{N}$ reaches its highest values in association with peak iron flux during the LGM.

Iron fertilization appears to dominate biological conditions, even over most of the millennial-scale climate changes. Between the two broad dust flux maxima of MIS 4 (57,000 to 71,000 years ago) and MIS 2 (14,000 to 29,000 years ago), there are four smaller but robust iron flux peaks observed

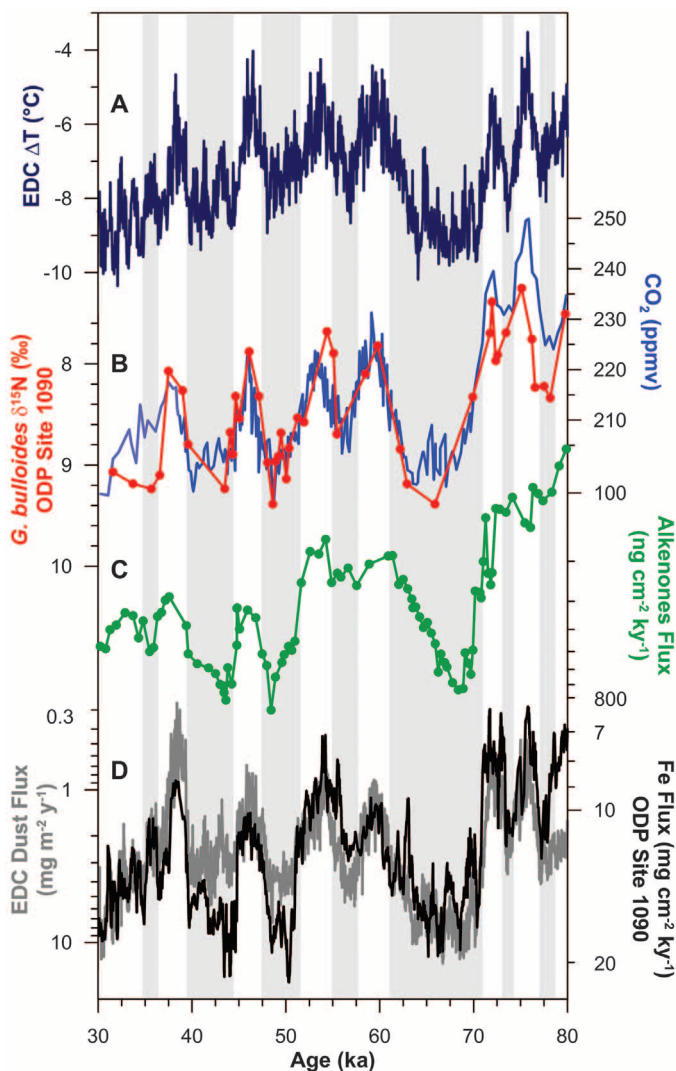
in both Antarctic ice and ODP Site 1090, which are associated with colder Antarctic temperature and lower atmospheric CO_2 (Fig. 3). Each of these peaks is associated with local maxima in $\text{FB-}\delta^{15}\text{N}$ and export production, as expected from iron fertilization.

Although the modest interglacial sedimentation rates at Site 1090 ($\sim 3 \text{ cm}$ per 1000 years) render the core nonideal for evaluating the sequence of events at deglaciations, the data suggest a tendency for $\text{FB-}\delta^{15}\text{N}$ to continue its deglacial decline after most of the decreases in dust and productivity (Fig. 2). Furthermore, in the early stages of the last ice age, $\text{FB-}\delta^{15}\text{N}$ rises subtly without the typical accompaniment of a rise in alkenone flux. Thus, with regard to these time intervals in particular, influences other than iron fertilization on Subantarctic nitrate consumption and $\text{FB-}\delta^{15}\text{N}$ warrant consideration.

In general, two processes seem likely to have modified the degree of nitrate consumption (and thus $\text{FB-}\delta^{15}\text{N}$) at Site 1090, overlaying their effects on that of dust-borne iron fertilization. First, as described above, the Subantarctic Zone and its boundaries may have moved equatorward during the last ice age and poleward again into the interglacials (33, 34). A poleward migration of the Subantarctic Front upon deglacial warming would decrease the concentration and increase the $\delta^{15}\text{N}$ of the nitrate being supplied to Site 1090 (16), both of which would work to raise $\text{FB-}\delta^{15}\text{N}$ at a given rate of productivity and nitrate use. Thus, in the deglacial decrease in $\text{FB-}\delta^{15}\text{N}$ associated with the waning of iron fertilization, frontal migration may yield pauses and/or reversals. At the initiation of glacial cooling, the reverse dynamic of increasing iron fertilization but equatorward movement of the fronts might apply.

Second, there is evidence that the degree of nitrate consumption in Antarctic surface waters rose during the ice ages (25), and this region represents one of the nitrate sources to the Subantarctic in the modern ocean (20). Thus, the Subantarctic $\delta^{15}\text{N}$ changes may include an effect from an increased nitrate $\delta^{15}\text{N}$ and/or decreased nitrate concentration in the Antarctic (29). The many instances of coupled $\text{FB-}\delta^{15}\text{N}$ and productivity rise are not solely due to an increase in the $\delta^{15}\text{N}$ or a decrease in concentration of the nitrate imported from the Antarctic, as neither would explain the Subantarctic productivity increases. Conversely, during the lowest dust flux intervals within the glacial sections at Site 1090, $\text{FB-}\delta^{15}\text{N}$ declines to near-interglacial levels, which suggests that the Subantarctic $\text{FB-}\delta^{15}\text{N}$ increase due to Antarctic nitrate consumption change was not more than $\sim 1\%$. These observations are consistent with our analysis that increased Antarctic nitrate consumption would raise only weakly the $\delta^{15}\text{N}$ of total nitrate supply to the Subantarctic, because a decline in Antarctic surface nitrate concentration would reduce its isotopic influence on the Subantarctic (29) (fig. S8). Perhaps the interval most likely to show the influence of Antarctic nitrate $\delta^{15}\text{N}$ change alone is the period from

Fig. 3. Millennial-scale coupling of Subantarctic iron flux and biogeochemistry with climate and atmospheric CO_2 . (A) EDC δD -based Antarctic air temperature reconstruction (45). (B) Atmospheric CO_2 concentration measured in Antarctic ice cores (38, 42) (blue line) and *G. bulloides* $\text{FB-}\delta^{15}\text{N}$ at ODP Site 1090 (red circles). (C) ^{230}Th -normalized alkenone flux from TN057-6 [parallel core to ODP Site 1090 (29)]. (D) EDC dust flux (gray line) (44) and ^{230}Th -normalized iron flux at ODP Site 1090 (black line). Here, *G. bulloides* $\text{FB-}\delta^{15}\text{N}$ and the fluxes of alkenones, iron, and dust all increase downward to facilitate the comparison with Antarctic air temperature and atmospheric CO_2 , and the fluxes are plotted on logarithmic scales. The gray shaded vertical bars highlight maxima in dust flux that correspond to minima in atmospheric CO_2 and Antarctic air temperature.



~110,000 to ~85,000 years ago, during which $\text{FB-}\delta^{15}\text{N}$ rises but there is no clear sign of an increase in Subantarctic productivity (Fig. 2).

The coincidence of the dust and productivity increases with the latter half of the CO_2 drawdown over the last ice age has been widely noted (15, 17, 35). Model simulations suggest that iron-driven drawdown of major nutrients in the Subantarctic can drive this 40-ppm portion of the ice age CO_2 decline without violating other constraints, such as those involving deep ocean calcite saturation state and the $^{13}\text{C}/^{12}\text{C}$ ratio of dissolved inorganic carbon (12). Our confirmation of iron fertilization in MIS 6 (prior to 130,000 years in our data) and in MIS 4–MIS 2 validates this long-held hypothesis for glacial-interglacial CO_2 change. The nearly complete nitrate drawdown estimated for Site 1090 is actually greater than required to explain 40 ppm of CO_2 decline, were this location representative of global Subantarctic Mode Water formation (24). However, Subantarctic Mode Water forms further south near the Subantarctic Front (36), where the peak ice age nitrate concentration may have been higher. Moreover, the Pacific sector of the Subantarctic may have experienced weaker iron fertilization, given lower ice age dust fluxes there (37). Nonetheless, we expect that ice age Subantarctic $\text{FB-}\delta^{15}\text{N}$ elevation applies to all sectors of the Southern Ocean because, even with strong zonal changes in dust deposition, the rapid eastward flow of the Circumpolar Current would weaken zonal gradients in nitrate concentration and $\delta^{15}\text{N}$. This expectation appears to be consistent with the available $\text{DB-}\delta^{15}\text{N}$ data (24).

With improvements in the ice core reconstructions, it has become clear that each of the millennial cold spells in Antarctica was associated with both an increase in dust flux to Antarctica and a decline in atmospheric CO_2 (Fig. 3). Our results indicate that these millennial-scale events were also associated with higher dust flux to the Atlantic Subantarctic, higher productivity, and fi-

nally more complete nitrate consumption (Fig. 3). Although attention has recently been focused on Antarctic overturning changes as the underlying cause of the millennial-scale CO_2 changes (38, 39), the Site 1090 data suggest that Subantarctic iron fertilization can also explain them. This raises the possibility that the millennial-scale CO_2 oscillations are caused by the two Southern Ocean mechanisms working in concert; a similar mechanism has been proposed to achieve the full ice age drawdown in atmospheric CO_2 (13).

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Supplementary Materials

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Materials and Methods
Figs. S1 to S9
References (46–66)

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From Parasitism to Mutualism: Unexpected Interactions Between a Cuckoo and Its Host

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Avian brood parasites lay eggs in the nests of other birds, which raise the unrelated chicks and typically suffer partial or complete loss of their own brood. However, carrion crows *Corvus corone corone* can benefit from parasitism by the great spotted cuckoo *Clamator glandarius*. Parasitized nests have lower rates of predation-induced failure due to production of a repellent secretion by cuckoo chicks, but among nests that are successful, those with cuckoo chicks fledge fewer crows. The outcome of these counterbalancing effects fluctuates between parasitism and mutualism each season, depending on the intensity of predation pressure.

Interspecific avian brood parasites generally harm their hosts in two main ways: Evicting parasites eject all other eggs and hatchlings

from the nest, whereas nonevicting parasites are raised alongside host offspring but usually outcompete some or all of them for food (1).

Specific defenses against brood parasites, including ejection of alien eggs and mobbing of parasitic adults (2), have evolved in many but not all host species (3). It has been hypothesized that lack of defenses may be due to relatively recent contact between the antagonistic species or to hosts refraining from exhibiting their defenses when costs outweigh benefits (1). Alternatively, defenses might not evolve if brood parasite-host interactions can switch to a mutualism, as suggested by Smith (4). His results from a study on giant cowbirds (*Scaphidura oryzivora*),

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however, were never replicated (5), and brood parasite-host systems still represent a paradigm of parasitic interactions. Here we show, in a system different from Smith's, that a brood parasite can indeed provide a benefit to its host.

The great spotted cuckoo (*Clamator glandarius*) is a nonevicting brood parasite specialized on corvids, mainly magpies (*Pica pica*) and carrion crows (*Corvus corone corone*) (6, 7). Cuckoos strongly reduce magpie reproductive success (6), but apparently this does not occur in crows, whose larger offspring are often raised alongside the parasite (7, 8). Unlike magpies, crow parents do not evict alien eggs or mob parasitic adults in proximity of the nest (9). In our study area in northern Spain (42°37'N, 5°26'W), the great spotted cuckoo can parasitize up to 67.7% of crow nests (8).

We investigated (i) whether the great spotted cuckoo provides a benefit to crows and whether such benefit could derive from the ability of cuckoo chicks to deter predators with a noxious secretion that they release when harassed, as well as (ii) whether the outcome of host-parasite interaction varies across seasons, depending on the intensity of predator pressure. To do so, we used data collected over 16 years to analyze the effect of the parasite on crow reproductive success. In the studied population, crows breed cooperatively and raise a single annual brood, although they can renest in case of early nest failure (10, 11). Each season, nests were monitored to record laying date, clutch size, presence of parasitic eggs, hatching success, and number of fledglings produced ($n = 741$ nests in 109 territories) (11). To test whether crow clutches benefit from being parasitized, we transferred cuckoo hatchlings (one or two per nest) into synchronous nonparasitized nests (average difference in laying date of the first egg $\pm$ SE = 0.54 ± 0.23 days), whereas unmanipulated parasitized and nonparasitized nests served as controls (11). Furthermore, to control for the effect of the manipulation (adding or removing chicks), in a subsequent

experiment we moved one crow chick between synchronous nonparasitized nests and kept unmanipulated nests for control (11). Finally, we used gas chromatography and mass spectrometry to analyze the chemical composition of cuckoo cloacal secretions, and we performed repellence tests on species belonging to the three main groups of crow nest predators (that is, mammals, corvids, and raptor birds) (11, 12). Seventeen quasi-feral cats (that is, free-ranging cats that hunt year-round but could be attracted with food) were each presented with 10 pieces of chicken meat, treated either with water or natural cuckoo secretion. Seven captive crows and seven captive raptors were each offered six pieces of meat (three treated and three control), one at a time.

Our analyses of the long-term data set (11) show that the seeming lack of cost of raising cuckoos on crow reproductive success resulted from the combination of two counterbalancing effects. Parasitized and nonparasitized nests had similar probability of reaching the hatching stage [0.771 and 0.731, respectively; z score (z) = 0.264, $P = 0.792$, $n = 741$] (table S1). Once the eggs hatched, parasitized nests were more successful (that is, more likely to produce at least one crow fledgling) as compared with nonparasitized nests (probability of success = 0.764 and 0.538, respectively; $z = 2.94$, $P = 0.003$, $n = 550$) (table S1). However, among nests that were successful, those containing a cuckoo chick produced fewer crow fledglings than those without cuckoos (average $\pm$ SE = 2.073 ± 0.139 and 2.564 ± 0.064 , respectively; $z = -2.670$, $P = 0.008$, $n = 312$) (table S1). Overall, throughout the 16 seasons, parasitized and nonparasitized broods did not significantly differ in the number of crows fledged (1.584 ± 0.149 versus 1.379 ± 0.068 , respectively; $z = 0.390$, $P = 0.694$, $n = 550$), though results suggest a slight benefit from raising a cuckoo.

The results of the translocation experiment show a causal link between the presence of a parasitic chick and greater nest success. Among parasitized nests, those from which cuckoos were

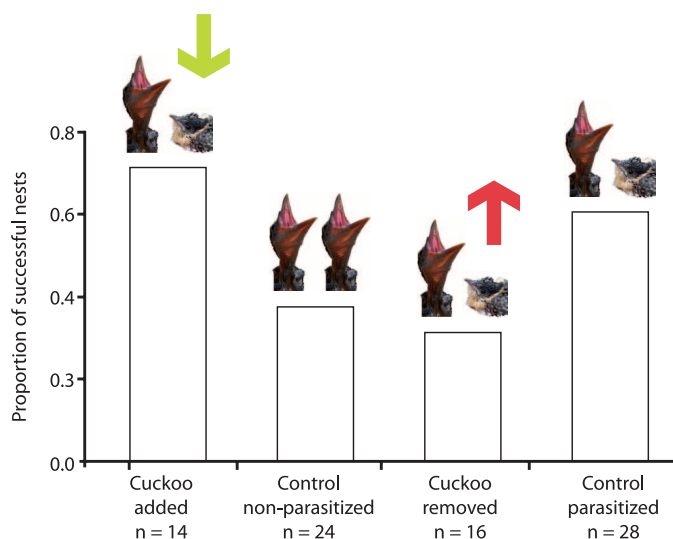
removed failed significantly more often than control nests (probability of success = 0.312 and 0.607, respectively; $z = -2.065$, $P = 0.039$) (Fig. 1 and table S2), whereas among nonparasitized nests, the addition of cuckoo chicks resulted in significantly increased success (unmanipulated nonparasitized = 0.375, cuckoo added = 0.714; $z = 1.984$, $P = 0.047$) (Fig. 1 and table S2). In sharp contrast to the effect of cuckoo chick transfer, nests in which a crow chick was added showed no difference in success rate (0.364, $n = 11$) from those that were not manipulated or those from which a crow chick was removed (0.818 and 0.727, respectively; $n = 11$ for both treatments; $z = 1.707$, $P = 0.088$ and $z = 1.785$, $P = 0.074$) (table S2), though the difference was suggestive of a reduction.

The most plausible mechanism driving the reduction of failure in nests with cuckoos is predator repellence by a malodorous cloacal secretion that parasitic chicks void when grabbed (supplementary text). This secretion is only produced by cuckoo nestlings (0 of 23 captured adults showed it) and can be copious (up to 1.2 ml released by a 45-g chick; average $\pm$ SE = 0.93 ± 0.06 ml, $n = 8$). When handling chicks, we observed voiding of the secretion in 20.8% of hatchlings (1 to 2 days old, $n = 24$), 71% of nestlings of 3 to 4 days ($n = 21$), and 90% of chicks older than 4 days ($n = 59$). At fledging, voiding of the secretion became less frequent (only two of six handled fledglings produced it).

The chemical analysis of cuckoo secretion revealed a mix of caustic and repulsive compounds, dominated by acids, indoles, phenols, and several sulfur containing compounds (Fig. 2) that are known to repel mammals and birds (13–16). Further chemical analyses confirmed the distinct volatile profile of a cuckoo's secretion as compared with feces of both cuckoos and crows, and its defensive function was confirmed by repellence tests. Eight of nine cats ate all 10 pieces of control meat, whereas only one of eight cats took a bite from treated meat (Fisher's Exact test, $P = 0.01$). When we reversed the treatment for 9 of the original 17 cats, those offered control meat ($n = 5$) ate all the pieces, but none bit the treated meat ($n = 4$; Fisher's Exact test, $P < 0.01$). Crows also showed avoidance of the treated meat (proportion of eaten/cached control and treated items = 0.524 and 0.150, respectively; $z = -2.432$, $P = 0.008$, $n = 7$) (table S3), as did the raptors (proportion of eaten control and treated items = 0.952 and 0.286, respectively; $z = -3.355$, $P = 0.001$) (table S3).

Our study area hosts a large community of avian and mammalian nest predators (11) that causes failure of 21.2 to 78% of breeding attempts annually (10). In our experimental sample, unmistakable signs of predation (presence of broken feathers and/or damage of nest lining) were found in 10 of 21 nests that were inspected closely after failure. Overall, the analyses of long-term data, the translocation experiment, the repellence tests,

Fig. 1. Probability of success of experimental and control nests.
n, number of nests.



and the chemical analyses imply that the cuckoo contributes to nest success by repelling predators. We believe that the outcome of this parasite-host interaction may depend on predator pressure and thus fluctuates among parasitism, commensalism, and mutualism. Long-term data fit this condition-dependent scenario. Whereas cuckoos decrease host reproductive success at low rates of nest failure (which is a suitable proxy of nest predation rate) (17), parasitized nests produced more fledglings than nonparasitized nests during breeding seasons with high nest predation (Spearman's rank correlation coefficient $R = 0.834$, $n = 16$, $P < 0.001$) (Fig. 3).

It can be argued that provisioning the cuckoo chicks may still harm the crow host by worsening the quality of its own offspring or by demanding for an exaggerated amount of care, and that these costs eventually outbalance any benefit that the parasite can provide. However, we found that (i) crow fledglings raised alongside cuckoos were not in poorer conditions compared with those brought up in absence of parasites (weight/tarsus³ $\pm$ SE with cuckoos = 2.06 ± 0.009 , without cuckoos = 2.01 ± 0.004 ; $t = 1.178$, $df = 315$, $P = 0.240$, $n = 386$) (table S4), and (ii) raising a

cuckoo chick required substantially less effort than rearing a crow chick, as can be expected given the smaller size of the parasitic nestlings (approximately one-third of the weight of a crow) (18). After controlling for total brood size (crows and cuckoos), broods containing cuckoo chicks required significantly fewer visits per hour than nonparasitized broods ($t = -2.637$, $df = 20$, $P = 0.016$, $n = 27$) (table S5). In addition, the total dependence period (nestling and postfledging) is shorter for cuckoos (19) than for crows (20). The analysis of data for 89 crow parents (61 males and 28 females) confirmed that raising cuckoos had no significant consequences on annual adult survival (probability of survival of parasitized adults = 0.821, nonparasitized = 0.727; $z = 1.355$, $P = 0.175$) (table S6). Moreover, parasitized parents did not have significantly lower reproductive success in the following year (average number of fledglings $\pm$ SE = 0.818 ± 0.234 and 0.848 ± 0.110 for parasitized and nonparasitized adults, respectively; $z = -0.250$, $P = 0.803$, $n = 45$ males and 18 females) (table S7).

It has been advocated that interspecific interactions should not be strictly categorized as parasitism, commensalism, or mutualism (21),

because costs and benefits for each partner may vary in space and time, producing variable outcomes depending on the environmental context [for example, seed predation and dispersal (22) and cleaning symbioses (23)]. Here, we demonstrated that the consequences of brood parasitism can be beneficial (supplementary text) and that this benefit may be context-dependent, possibly preventing the evolution of host defenses, particularly when a nonvicting cuckoo parasitizes a larger host.

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Supplementary Materials

www.sciencemag.org/content/343/6177/1350/suppl/DC1
Materials and Methods
Supplementary Text
Tables S1 to S9
References (24–27)

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Fig. 2. Chromatogram of the volatiles of cuckoo nestling secretion. 1, acetic acid; 2, propanoic acid; 3, dimethyl disulfide; 4, isobutyric acid; 5, butyric acid; 6, pivalic acid; 7, isovaleric acid; 8, 2-methylbutanoic acid; 9, valeric acid; 10, α -pinene; 11, dimethyl trisulfide; 12, phenol; 13, caproic acid; 14, 3-carene; 15, acetophenone; 16, p-cresol; 17, 2-nonanone; 18, camphor; 19, dimethyl tetrasulfide; 20, indole; 21, skatole; 22, 2-dodecen-1-ol; 23, cyclic hexaatomic sulfur; 24, geranyl phenylacetate; 25, 2-tridecanone. Asterisks denote air contaminants, plasticizers, etc. Relative abundance is measured in number of ions.

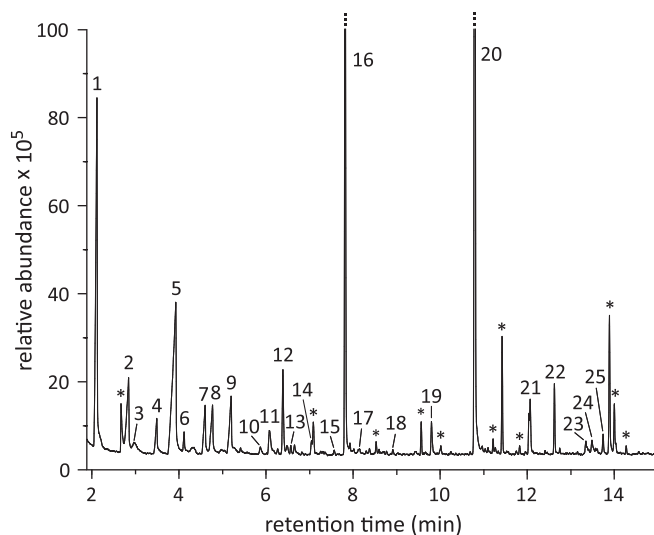
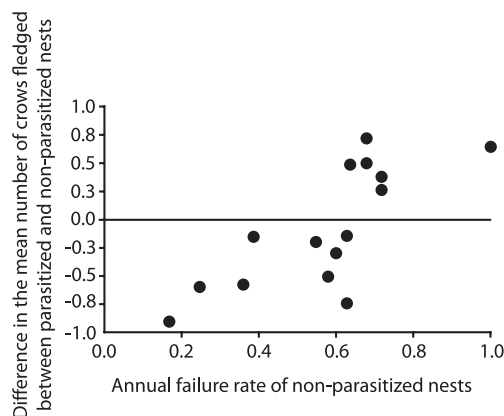


Fig. 3. Annual difference in the mean number of crows fledged between parasitized and nonparasitized nests plotted against annual failure rate of nonparasitized nests (proxy of nest predation rate). Above the zero line, the host benefits from the presence of the cuckoo.



β -Catenin Activation Regulates Tissue Growth Non–Cell Autonomously in the Hair Stem Cell Niche

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Wnt/ β -catenin signaling is critical for tissue regeneration. However, it is unclear how β -catenin controls stem cell behaviors to coordinate organized growth. Using live imaging, we show that activation of β -catenin specifically within mouse hair follicle stem cells generates new hair growth through oriented cell divisions and cellular displacement. β -Catenin activation is sufficient to induce hair growth independently of mesenchymal dermal papilla niche signals normally required for hair regeneration. Wild-type cells are co-opted into new hair growths by β -catenin mutant cells, which non–cell autonomously activate Wnt signaling within the neighboring wild-type cells via Wnt ligands. This study demonstrates a mechanism by which Wnt/ β -catenin signaling controls stem cell–dependent tissue growth non–cell autonomously and advances our understanding of the mechanisms that drive coordinated regeneration.

Adult tissue regeneration relies on the coordinated activation of resident stem cells within their niches to generate a functional organ (1). The Wnt/ β -catenin signaling pathway functions in the maintenance of stem

cells (SCs) of various lineages. However, it is unclear how this pathway controls specific subpopulations of cells to organize growth during tissue regeneration.

The hair follicle (HF) serves as a versatile model to address this fundamental question because it is highly accessible and its SCs and progeny, located in the bulge and hair germ, respectively, are anatomically and molecularly well-defined (fig. S1) (2–6). HF stem cell (HF-SC) progeny lie in direct contact with a specialized group of mesenchymal cells, the dermal papillae (DPs), that functions as a key signaling center required for

epithelial-mesenchymal interactions that govern HF growth (4, 7, 8).

Wnt signaling is required for HF development and regeneration (9–17) and is mediated by the stabilization and translocation of the key signal transducer, β -catenin, to the nucleus, where it binds TCF/LEF transcription factors to activate Wnt target gene transcription (18). Expression of activated β -catenin throughout the basal epidermis has been shown to induce de novo HFs within the epidermis, which establishes that Wnt signaling is required and sufficient for new hair growth (13, 15–17). Although these studies illustrate the importance of Wnt/ β -catenin signaling during HF regeneration, several outstanding questions remain, including (i) which dynamic SC behaviors does Wnt/ β -catenin signaling regulate, (ii) is Wnt/ β -catenin activation sufficient to promote growth within the SC pool independently of the mesenchymal DP cells, and (iii) what are the molecular mechanisms by which Wnt/ β -catenin coordinates collective tissue growth?

To investigate HF-SC behaviors regulated by Wnt/ β -catenin signaling, we genetically activated β -catenin specifically within the HF-SCs and their progeny using *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}* mice (Fig. 1A and fig. S1). Tamoxifen induction during the HF resting phase results in β -catenin stabilization and constitutive activation of Wnt signaling with subsequent formation of new ectopic axes of hair growth that show HF differentiation (Fig. 1A and figs. S1 and S2, A to F). These data show that β -catenin activation specifically in HF-SCs and their progeny can induce new hair growths, despite previous studies

Fig. 1. Activated β -catenin–induced cellular mechanisms that promote new hair growths.

(A) Hair growths in tamoxifen-treated *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}* mice. New hair growths are P-cadherin⁺ (green) and proliferative (Ki-67, red). Nuclei [4',6'-diamidino-2-phenylindole (DAPI), blue]. (B) Optical sections of z stacks of a *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}; K14-H2B-GFP* HF depict upward nuclear movement (movie S2). Epithelial nuclei (K14-H2B-GFP, white). Arrowheads indicate points of reference of original nuclei positions. Colored circles represent moving nuclei; black circles represent stationary nuclei. (C) Formation (c' inset) and expansion (c'') of nuclear clusters in two separate *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}; K14-H2B-GFP* mice. Nuclei are pseudocolored (movies S3 and S4). (D) *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}; K14-H2B-GFP* HFs just after DP ablations (day 0, yellow arrows). DPs marked by Lef1-RFP (red), epithelial nuclei by K14-H2B-GFP (green). (E) Same HFs after 6 days of tamoxifen treatment (day 7). (F and G) Revisit of the same HFs (days 12 to 19). Asterisk denotes HF out of plane of view. Scale bars, 25 μ m.

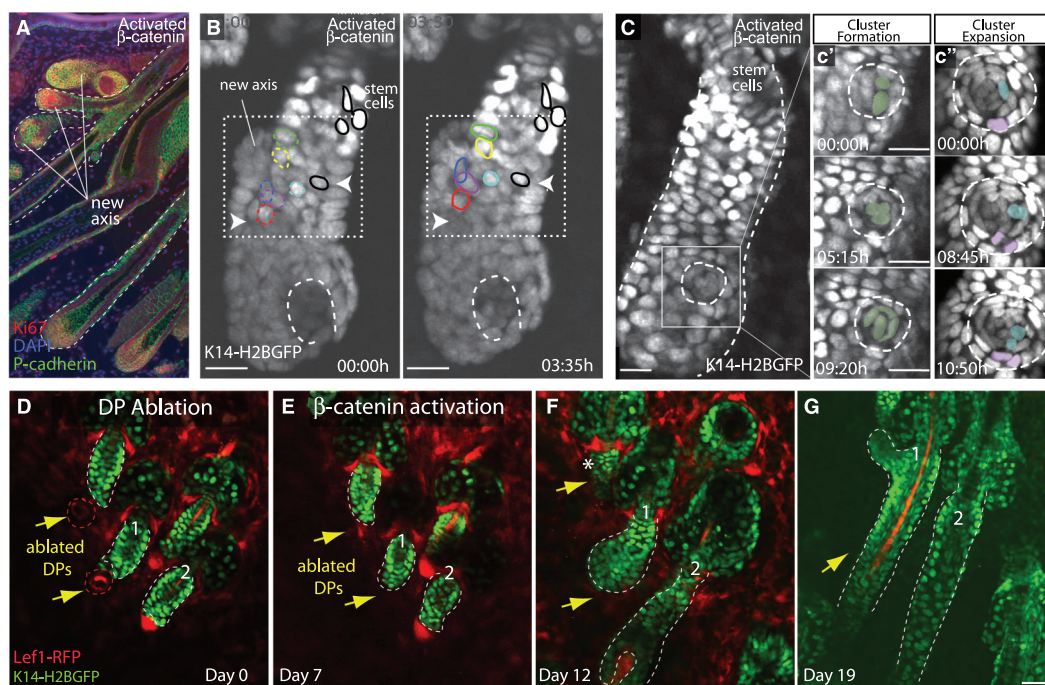


Fig. 2. β -Catenin activation triggers new axes composed of both mutant and wild-type cells. (A and B) Optical sections of z stacks of *K19Cre^{ER}; β -catn^{flox(Ex3)/+}; K14-H2B-GFP; tdTom* mutant HFs show early and late hair growths (K14-H2B-GFP, green; tdTom Cre reporter, red). (C) Genotype of FACS-isolated populations from the new growths based on P-cadherin enrichment (top gel) ($n = 2$ mice) or *TCF/Lef:H2B-GFP* Wnt reporter (bottom gel) ($n = 2$ mice). (D) Both tdTom⁺ and tdTom⁻ cells dividing within a new growth. Schematic depicts representative divisions within both tdTom⁺ and tdTom⁻ populations over a time-lapse recording (see movie S5). Scale bars, 50 μ m.

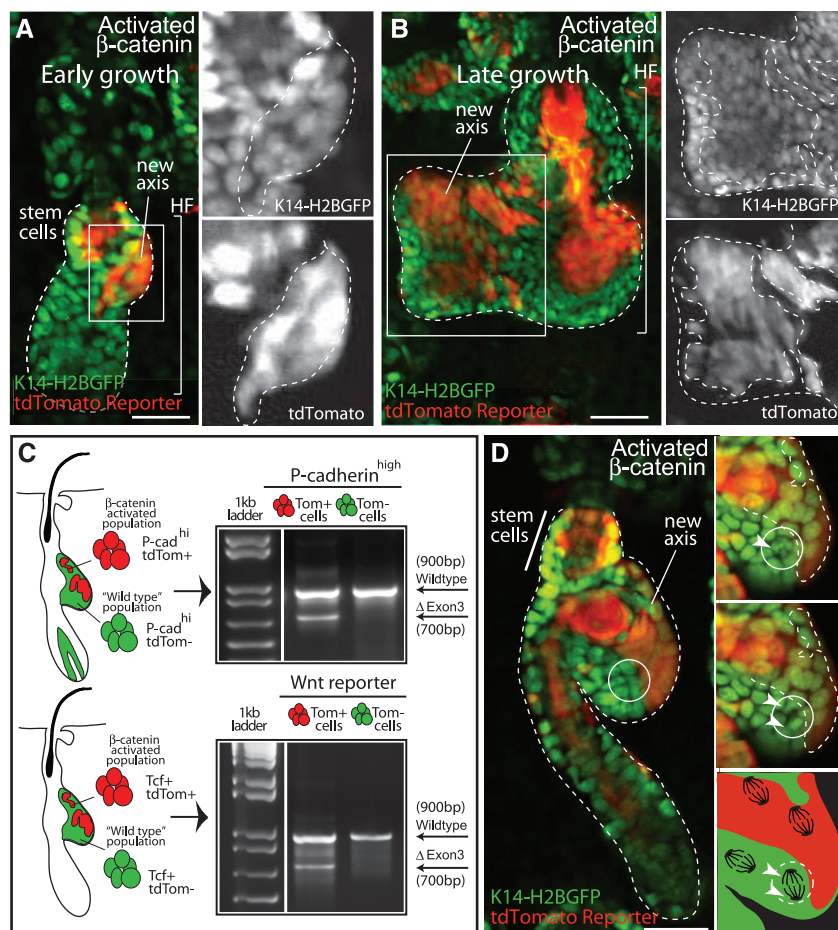
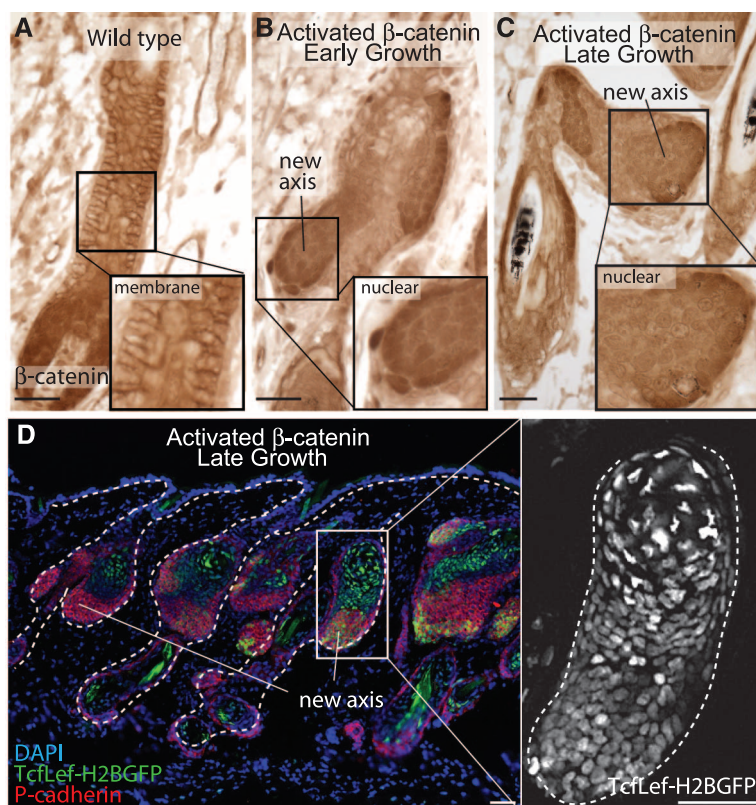


Fig. 3. β -Catenin mutant cells activate Wnt signaling in neighboring wild-type cells. (A) Immunohistochemistry of β -catenin in growing wild-type HF. Inset highlights membrane-bound staining. (B and C) Nuclear β -catenin in new hair growths in tamoxifen-induced *K19CreER; β -catn^{flox(Ex3)/+}* mice at early and late growth stages. Insets highlight nuclear staining. (D) Wnt reporter expression (*TCF/Lef:H2B-GFP*, green) in late growths (P-cadherin, red). Scale bars, 50 μ m.



that suggested that HF-SCs and progeny cells might be refractory to activated β -catenin signaling relative to other epidermal keratinocytes (16, 19). In contrast to ectopic HFs that form when β -catenin is activated throughout the basal epidermis (14, 16), new hair growths in $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+}$ mice did not harbor morphologically apparent DP structures but, instead, were surrounded by a layer of mesenchymal cells that expressed DPs or dermal sheath markers (fig. S2, G to J), consistent with previous findings (20).

Next, we coupled our genetic gain-of-function system with in vivo imaging of live mice. Time-lapse imaging of new hair growth in $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};K14\text{-H2B-GFP}$ mice [in which green fluorescent protein (GFP) highlights all skin epithelial cells] captured cell divisions that were oriented along the new axis of growth (fig. S3, A to D) and displayed divergent upward displacement of epithelial nuclei toward newly forming hair growths (Fig. 1B and movies S1 and S2). These budlike clusters of epithelial cells organized themselves into a compact arrangement (fig. S3E and movie S2). To capture early changes induced by β -catenin stabilization, we

began recording when mutant follicles had not yet developed new hair growths and observed epithelial nuclei clustering into ringlike structures [Fig. 1C (top view), fig. S3E (side view), and movies S3 and S4], reminiscent of early-stage embryonic HF formation (fig. S4) (21). These findings show that activated β -catenin orients cell divisions and organizes cell movements within SC progeny cells to drive new axes of hair growth.

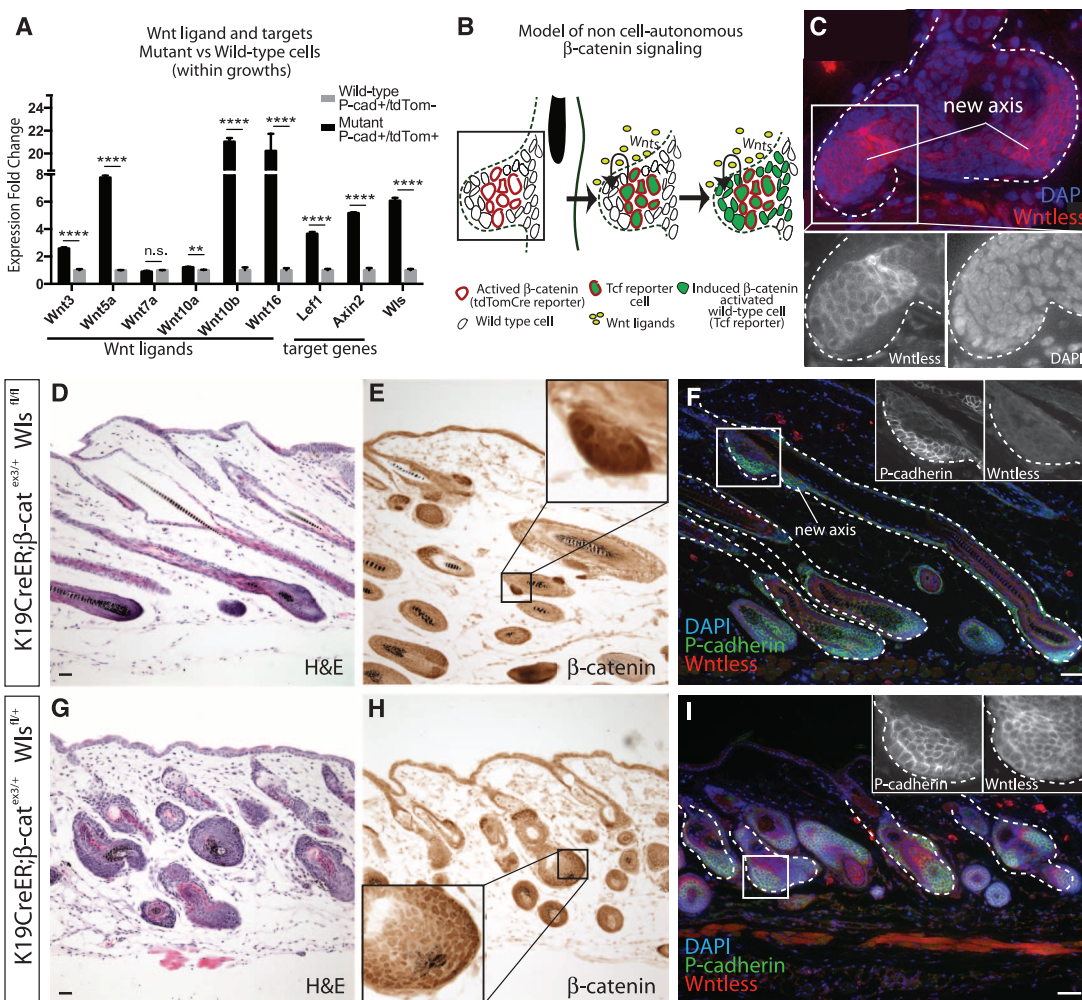
The mesenchymal DP constitutes one of the best-characterized niches for HF-SCs and their progeny and are required for their activation to initiate hair growth (8, 22, 23). Therefore, we hypothesized that native DP signals may be required parallel or downstream of activated β -catenin in HF-SC progeny cells to initiate new hair growths. To test this hypothesis in vivo, we first laser-ablated DP cells and then induced β -catenin activation in HF-SCs and progeny using $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};K14\text{-H2B-GFP};Lef1\text{-RFP}$ mice (7) [in which red fluorescent protein (RFP) highlights DP cells]. We then revisited the same ablated follicles over several days (Fig. 1, D to G, and fig. S5). The majority of β -catenin mutant HFs regrew (62%) in contrast to wild-type

HFs (0%) after DP ablation (fig. S5F). Although we cannot exclude a potential requirement of a native DP for subsequent HF differentiation or that other mesenchymal cells may functionally promote hair growth in the absence of a native DP, these data demonstrate that β -catenin activation in the SC and progeny compartments is sufficient to initiate HF growth independent of mesenchymal DP niche signals.

To address the mechanism by which mutant β -catenin-activated cells contribute to hair growth expansion, we labeled mutant cells using a tdTomato Cre reporter (24). With in vivo imaging, we examined the contribution of tdTomato-positive cells (tdTom⁺) to the new hair growths in tamoxifen-induced $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};K14\text{-H2B-GFP};tdTom$ mice. Although the initial small hair growths largely consisted of tdTom⁺ cells, later expanded growths were mosaic for tdTom expression, which suggests that mutant cells recruit wild-type cells to promote growth and expansion (Fig. 2, A and B, and fig. S6A) and are reminiscent of the mosaic contribution observed in tooth formation after β -catenin activation (25). To confirm this heterogeneity, we purified cells from growths by fluorescence-activated cell sorting

Fig. 4. β -Catenin acts non-cell autonomously via Wnt ligands.

(A) Representative qRT-PCR analysis of Wnt ligands and target genes comparing sorted $Pcad^{H1};tdTom^{+}$ versus $Pcad^{H1};tdTom^{-}$ populations from $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};tdTom$ mice ($n = 2$; $P < 0.005$). (B) Model of non-cell autonomous β -catenin signaling. (C) Wntless staining (red) in $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+}$ mice. (D) After tamoxifen induction, hematoxylin and eosin (H&E) staining of $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/fl}$ mice shows anagen-like stage HFs with growths of varying sizes versus (G) $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/+}$ mutant controls, which show growths with a higher frequency and larger size ($n = 3$ experimental litters, $n = 7$ $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/fl}$ mice). (E) Nuclear β -catenin within growths of $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/fl}$ and controls (H). (F) Wntless (red) is absent in most of the growths of $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/fl}$ compared to mutant controls (I). Nuclei (DAPI, blue). Scale bars, 50 μ m.



(FACS) using tdTom and P-cadherin expression, a marker enriched in these growths. FACS-purified mutant (tdTom⁺;P-cad^{hi}) cells showed the expected deleted β -catenin- Δ Exon3 allele by polymerase chain reaction (PCR) [700-base pair (bp) band], whereas wild-type (tdTom⁻;P-cad^{hi}) cells showed only the nonrecombined band (900 bp) (Fig. 2C, top gel). In the same way, FACS purification using the *TCF/Lef:H2B-GFP* Wnt reporter (fig. S6B) in the *K19Cre^{ER};β-catn^{fllox(Ex3)/+};tdTom* mice showed similar results (Fig. 2C, bottom gel).

To determine whether wild-type cells functionally contribute to the expansion of mutant hair growths, we performed time-lapse recordings and immunofluorescent analysis and demonstrated that wild-type cells within the growths proliferate like coresident β -catenin mutant cells (Fig. 2D, fig. S6C, and movie S5). These results show that β -catenin activation in a subset of HF-SCs and their progeny leads to the recruitment of wild-type epithelial that collectively promote coordinated hair growth expansion.

On the basis of observed cellular heterogeneity, we predicted heterogeneous activation of the Wnt/ β -catenin pathway throughout the new growths. At early stages, nuclear β -catenin was observed within the induced hair growths (Fig. 3, A and B). As growths expanded, nuclear β -catenin was still present globally in the new growth axes. However, clusters of cells with stronger nuclear β -catenin staining neighbored cells that exhibited weaker nuclear β -catenin staining (Fig. 3C). Additionally, TCF/Lef:H2B-GFP and Lef1 immunohistochemistry showed that Wnt active cells were observed throughout the new hair axes (Fig. 3D and fig. S6), which suggests that both activated β -catenin mutant cells and wild-type cells initiate Wnt signaling.

To address how a mosaic population of cells can activate Wnt signaling, we first assessed global transcriptional changes in Wnt pathway genes by quantitative reverse transcription PCR (qRT-PCR) from whole skin of mutant and control littermate *K19Cre^{ER};β-catn^{fllox(Ex3)/+}* mice. Wnt target genes (*Axin2*, *CyclinD*, and *Lef1*) and several Wnt ligands were up-regulated in β -catenin mutant skin compared to that of control littermates (fig. S7A), consistent with previous studies that show up-regulation of Wnt ligands after β -catenin activation (26–29). To determine whether β -catenin-activated mutant cells specifically up-regulate Wnt ligand expression, we FACS-purified mutant cells (tdTom⁺;P-cad^{hi}) and found that several Wnt ligands (*Wnt3*, *5a*, *10b*, and *16*), as well as a gene required for Wnt ligand secretion (*Wntless*), were all up-regulated compared to wild-type (tdTom⁻;P-cad^{hi}) cells (Fig. 4, A to C). Furthermore, wild-type cells within the growths (tdTom⁻/P-cad^{hi}/K14-H2B-GFP⁺) showed higher expression of the direct downstream Wnt target gene, *Axin2*, than wild-type cells outside the growths (tdTom⁻/P-cad^{hi}/K14-H2B-GFP⁺) (fig. S7B). This shows that Wnt signaling is active in the wild-type cells inside the new hair growths when compared to

remaining follicular epithelial cells. Furthermore, in situ hybridization analysis of *Wnt10b* and *Wnt16* showed expression localized to foci within the new growths (fig. S8).

Altogether, these experiments suggest a model in which mutant β -catenin cells influence wild-type cells to proliferate and contribute to the formation of new growths via Wnt ligand secretion (Fig. 4B). To test whether Wnt ligand secretion by β -catenin-activated cells influences the growth of wild-type cells in a paracrine manner, we used an in vitro culture assay. Wild-type keratinocytes showed increased proliferation when cultured in the presence of conditioned media collected from mutant β -catenin-activated cell cultures. This effect was abrogated when using conditioned media obtained from mutant β -catenin-activated cells cultured in the presence of a specific inhibitor of Wnt ligand secretion, IWP2 (fig. S9, A to C). To test in vivo whether Wnt secretion contributes to the development or expansion of growths, we conditionally deleted *Wntless* (Wls) using *K19Cre^{ER};β-catn^{fllox(Ex3)/+};Wls^{fl/fl}* mice (30). Activated β -catenin-induced hair growths were largely delayed and overall reduced in size in the absence of Wls compared to β -catenin mutant control mice (*K19Cre^{ER};β-catn^{fllox(Ex3)/+};Wls^{fl/+}*) (Fig. 4, D to I). When larger growths were observed in the *K19Cre^{ER};β-catn^{fllox(Ex3)/+};Wls^{fl/fl}* mice, they were associated with expression of Wls, which suggests that the efficiency of Wls elimination was inversely related to aberrant growth (fig. S9, D to F). These data show that Wnt/ β -catenin signaling acts through a non-cell autonomous mechanism to support collective cellular growth, a model that may explain previous findings of mosaic contribution to other epithelial organs. Furthermore, these results demonstrate that Wnt ligand secretion is an important downstream mediator of β -catenin-induced non-cell autonomous tissue growth.

This study brings a more detailed understanding of how a conserved regenerative signal, Wnt/ β -catenin, temporally and spatially regulates SCs and progeny behaviors during tissue growth. These data highlight how a mutation in a downstream target gene can modify the behavior of a genetic clone of cells while also co-opting surrounding wild-type cells to acquire similar behaviors. These findings hold important implications for understanding the cellular mechanisms that promote coordinated tissue growth and regeneration.

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Supplementary Materials

www.sciencemag.org/content/343/6177/1353/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S9
Table S1
References (31–37)
Movies S1 to S5

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Activity of Protein Kinase RIPK3 Determines Whether Cells Die by Necroptosis or Apoptosis

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Receptor-interacting protein kinase 1 (RIPK1) and RIPK3 trigger pro-inflammatory cell death termed "necroptosis." Studies with RIPK3-deficient mice or the RIPK1 inhibitor necrostatin-1 suggest that necroptosis exacerbates pathology in many disease models. We engineered mice expressing catalytically inactive RIPK3 D161N or RIPK1 D138N to determine the need for the active kinase in the whole animal. Unexpectedly, RIPK3 D161N promoted lethal RIPK1- and caspase-8-dependent apoptosis. In contrast, mice expressing RIPK1 D138N were viable and, like RIPK3-deficient mice, resistant to tumor necrosis factor (TNF)-induced hypothermia. Cells expressing RIPK1 D138N were resistant to TNF-induced necroptosis, whereas TNF-induced signaling pathways promoting gene transcription were unperturbed. Our data indicate that the kinase activity of RIPK3 is essential for necroptosis but also governs whether a cell activates caspase-8 and dies by apoptosis.

Tumor necrosis factor receptor 1 (TNFR1), Toll-like receptors (TLRs), and antigen receptors trigger necroptotic cell death when caspase-8 is inhibited and cells express receptor-interacting protein kinase 3 (RIPK3) and its substrate MLKL (mixed lineage kinase domain-like) (1). Wild-type RIPK3, but not catalytically inactive RIPK3 mutants, can reconstitute necroptosis in RIPK3-deficient cells (2–4), indicating that the kinase activity of RIPK3 is critical. TNFR1 engages RIPK3 through RIPK1, whereas TRIF

(Toll/interleukin-1 receptor domain-containing adaptor inducing interferon- β) links RIPK3 to TLRs 3 and 4 (1). In the absence of antibodies that label cells dying by necroptosis, the contribution of necroptosis to pathology has been inferred from the resistance of *Ripk3*^{−/−} mice to pancreatitis, retinal degeneration, atherosclerosis, liver injury, and systemic inflammation (1). The caveat here is that loss of RIPK3 could indicate a scaffold function for RIPK3 rather than a requirement for its kinase activity. Necrostatin-1 (nec-1) inhibits RIPK1

and ameliorates disease in some animal models too, although nec-1 might also inhibit other cellular processes via inhibition of indoleamine 2,3 dioxygenase (5).

We sought genetic proof that the kinase activity of RIPK3 drives necroptosis and disease by generating *Ripk3* knock-in mice with Asp¹⁶¹ of the catalytically important DFG motif mutated to Asn (fig. S1, A and B). Whereas *Ripk3*^{−/−} mice are viable (6), *Ripk3*^{D161N/D161N} mice died around embryonic day 11.5 (E11.5) with abnormal yolk sac vasculature (Fig. 1, A and B). (Single-letter abbreviations for the amino acid residues are as follows: D, Asp; N, Asn. In the mutants, other amino acids were substituted at certain locations; for example, D161N indicates that aspartic acid at position 161 was replaced by asparagine.) Staining for the endothelial cell marker PECAM-1 (platelet/endothelial cell adhesion molecule 1) and cleaved caspase-3, a marker of apoptosis, revealed significantly increased apoptosis in the *Ripk3*^{D161N/D161N} yolk sac vasculature (Fig. 1C). RIPK3 is expressed in endothelial cells and gut epithelial cells during development (fig. S1C). *Ripk3*^{D161N/−} mice, which are expected

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Fig. 1. *Ripk3*^{D161N/D161N} mice die during embryogenesis. (A) Numbers of surviving offspring from *Ripk3*^{D161N/+} parents. Mouse strains were derived from independent *Ripk3*^{D161N/+} embryonic stem cell clones. (B) E11.5 embryos and their yolk sacs. (C) E11.5 yolk sacs stained for PECAM-1 (red) and cleaved caspase-3 (CC3) (green). Scale bar, 200 μ m. Each square in the scatter plot represents the yolk sac of one embryo. CC3⁺ cells were counted in 8 to 17 fields per embryo, and the mean number per field is plotted. *P* value determined with Student's *t* test.

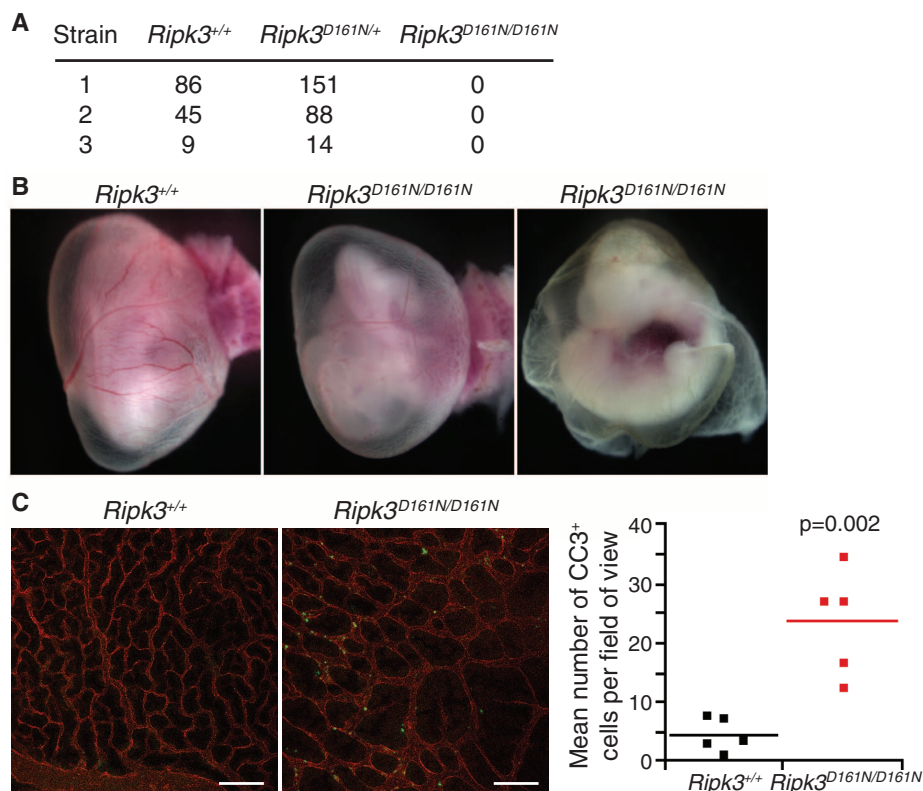


Fig. 2. RIPK1- and caspase-8–dependent embryonic lethality caused by RIPK3 D161N. (A) Proposed mechanism for control of necroptosis and apoptosis by RIPK3 and RIPK3 D161N. (B) Numbers of surviving offspring from *Casp8*^{+/+} *Ripk3*^{D161N/+} parents. (C) Spleen and lymph nodes from mice aged 16 to 20 weeks. Scale bar, 1 cm. (D) Leukocytes in the spleen or brachial, inguinal, mesenteric, and axillary lymph nodes were counted. Bars represent the mean ± SD. (E) Numbers of P3 to P6 offspring from *Ripk1*^{+/+} *Ripk3*^{D161N/+} parents. (F) E18.5 littermates.

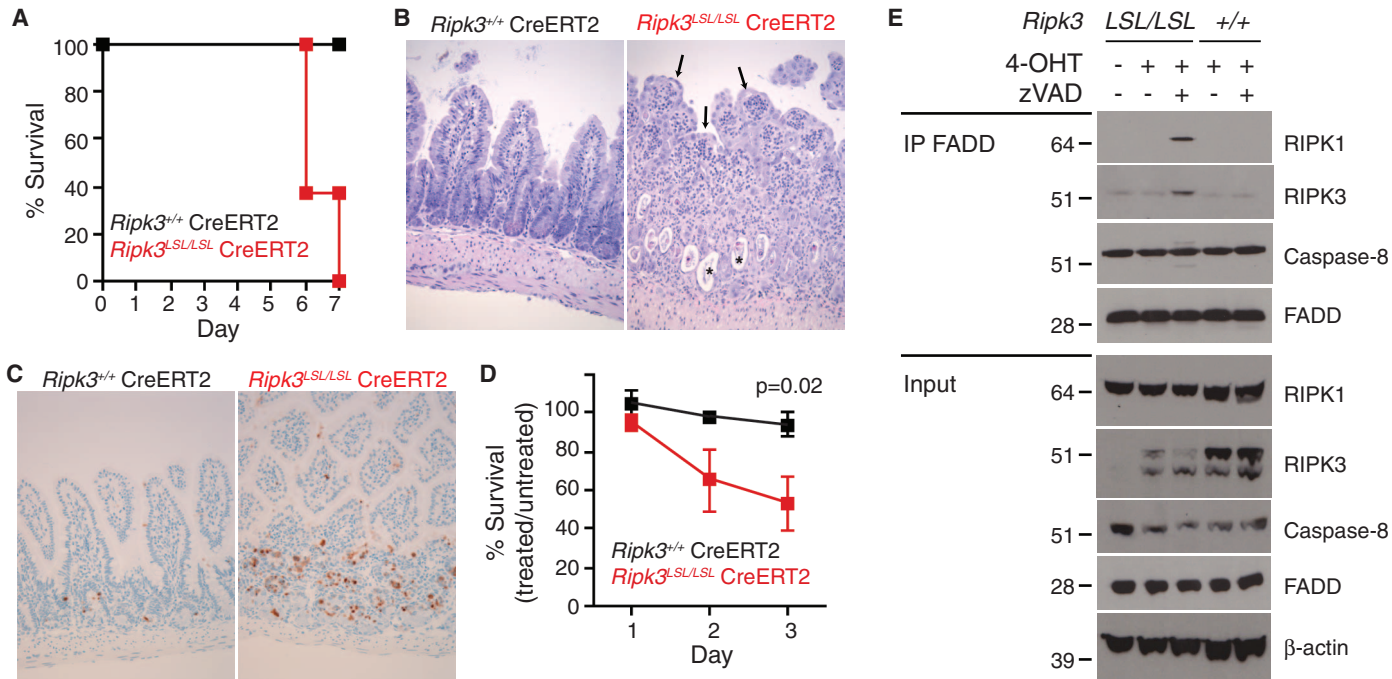
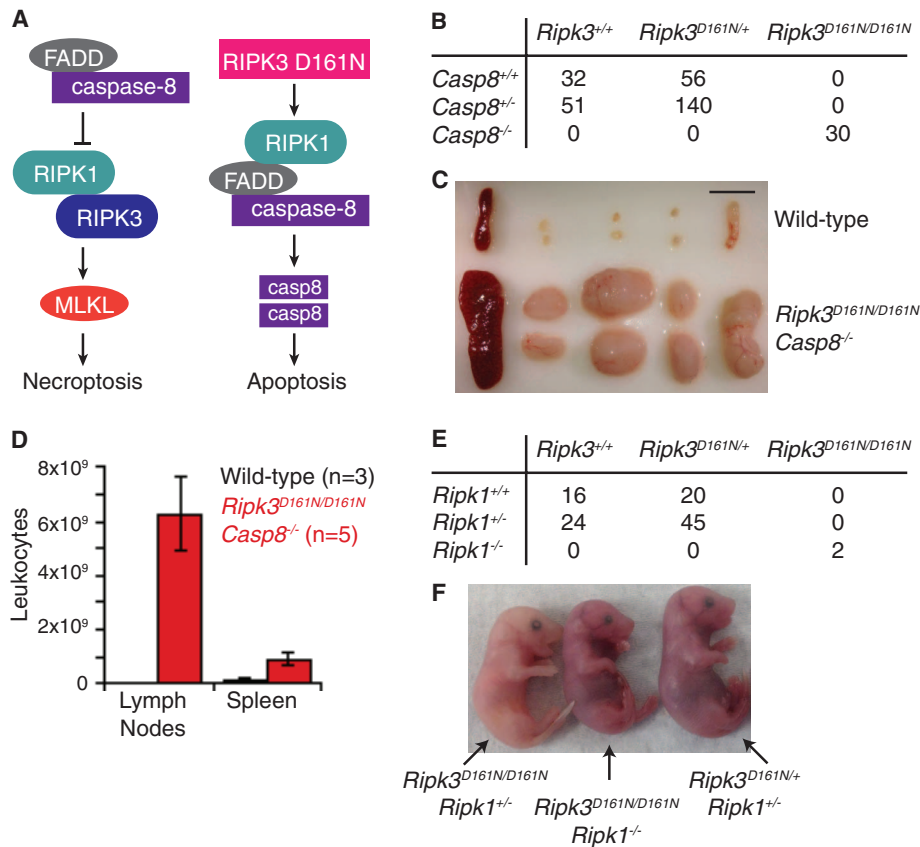


Fig. 3. Lethal effect of RIPK3 D161N expressed in adult mice. (A) Effect of tamoxifen-induced RIPK3 D161N expression on adult mouse survival. Mice ($n = 8$ per genotype) received daily tamoxifen injections on days 1 through 5. (B) Hematoxylin and eosin stained small intestine on day 7. Arrows indicate blunted and fused villi. Asterisks indicate crypt degeneration. Magnification is 20 $\times$. (C) Small intestine harvested on day 5. Cells expressing cleaved caspase-3 are stained brown. Magnification is 20 $\times$. (D) Survival of

MEFs cultured in 4-hydroxytamoxifen (4-OHT). Percent survival = $100 \times$ [percent viable cells (unstained by propidium iodide) after 4-OHT treatment]/[percent viable cells in medium alone]. Data represent the mean $\pm$ SD of three lines per genotype. P value on day 3 determined with Student's t test. (E) Western blots of FADD-containing protein complexes immunoprecipitated from CreERT2 MEFs cultured for 2 days in medium, 4-OHT, or 4-OHT and zVAD.fmk.

to make half the amount of RIPK3 D161N, were viable. Therefore, there may be a threshold above which the RIPK3 D161N protein becomes lethal. *Ripk3*^{D161N/+} mice had a normal life span, arguing against RIPK3 D161N acting as a dominant negative mutant.

The genetic evidence for caspase-8 and its adaptor FADD (Fas-associated via death domain) inhibiting necroptosis caused by RIPK1 and RIPK3 is compelling (1). We tested whether RIPK3 D161N promoted caspase-8-dependent apoptosis (Fig. 2A) by breeding the *Ripk3*^{D161N} allele onto a caspase-8-null background (fig. S2A). *Casp8*^{-/-} embryos, like *Ripk3*^{D161N/D161N} embryos, die mid-gestation (7), but double mutant *Casp8*^{-/-} *Ripk3*^{D161N/D161N} mice were viable (Fig. 2B). Halving the *Casp8* gene dosage delayed lethality until ~E15.5 (fig. S2B). *Casp8*^{-/-} *Ripk3*^{D161N/D161N} mice resembled *Casp8*^{-/-} *Ripk3*^{-/-} mice (8, 9) because they accumulated T cells expressing the surface markers CD3 and B220 (fig. S2C) and developed the lymphadenopathy and splenomegaly (Fig. 2, C and D) that is characteristic of impaired apoptosis signaling by the death receptor Fas. These data confirm that (i) RIPK3 D161N promotes caspase-8-dependent apoptosis during embryogenesis and (ii) the kinase activity of RIPK3 is required for the death of *Casp8*^{-/-} embryos. The kinase activity of RIPK3 also appears to mediate the proliferation defect of *Casp8*^{-/-} T cells (10) because *Casp8*^{-/-} *Ripk3*^{D161N/D161N}

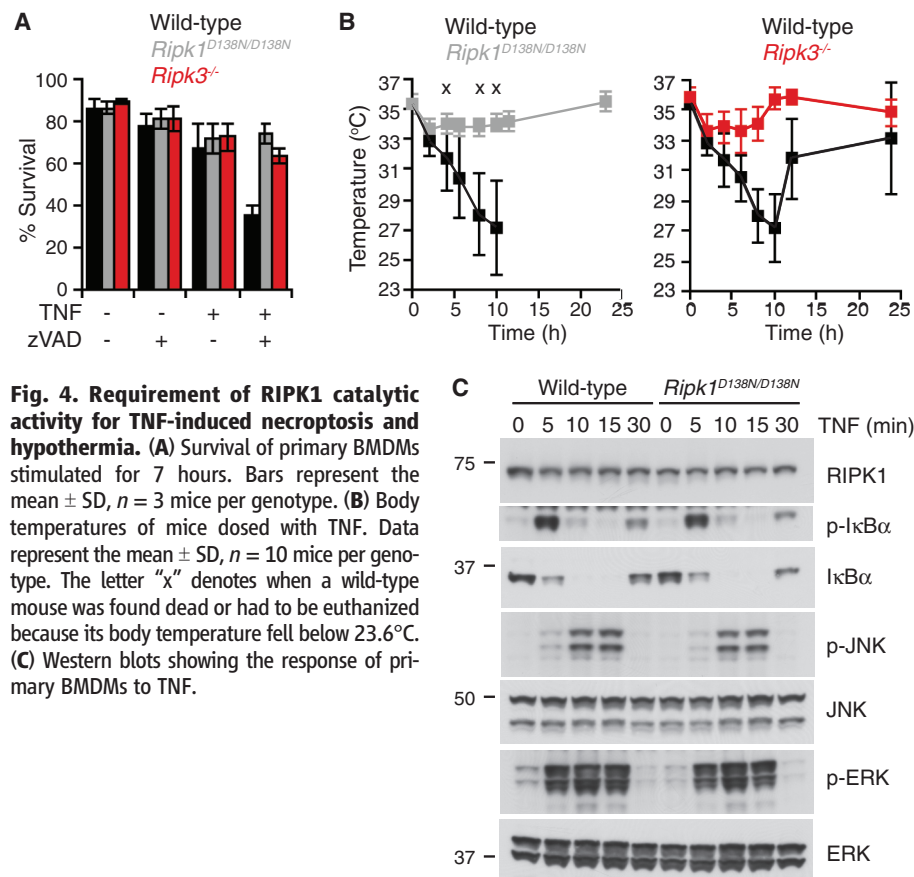
T cells proliferated normally in response to anti-bodies to CD3 and CD28 (fig. S2D).

Although a direct interaction between RIPK3 D161N and caspase-8 during embryogenesis is a possibility, we think that RIPK3 may influence caspase-8 through RIPK1 and FADD. RIPK3 and RIPK1 interact via their RIP homotypic interaction motifs (RHIMs) (11); RIPK1 and FADD can interact through their death domains (12); and FADD and caspase-8 interact through their death effector domains (13). Consistent with this model, RIPK1 deficiency (fig. S3A) rescued embryonic lethality caused by RIPK3 D161N (Fig. 2, E and F). *Ripk1*^{-/-} mice died soon after birth with increased cell death in the colon, brown adipose tissue, and thymus (fig. S3B). RIPK1 is abundant in the wild-type intestine at E18.5 and can also be detected in brown fat and liver (fig. S3C). *Ripk1*^{-/-} *Ripk3*^{D161N/D161N} mice died soon after birth with the same intestinal lesions as *Ripk1*^{-/-} mice (fig. S3B), suggesting that the defects caused by RIPK1 loss do not require RIPK3-dependent necroptosis. Halving the *Ripk1* gene dosage delayed death due to RIPK3 D161N (Fig. 2F). At E18.5, pale *Ripk1*^{+/-} *Ripk3*^{D161N/D161N} embryos exhibited none of the lesions associated with RIPK1 loss but contained fewer hematopoietic cells and less hepatic glycogen (fig. S3D). *Ripk3*^{D161N/D161N} embryos lacking MLKL, TRIF, the RHIM protein DAI (DNA-dependent activator of interferon-regulatory factors), the RIPK1 de-

ubiquitinase CYLD (cylindromatosis), TNFR1, DR3 (death receptor 3), or FLIP (FLICE-inhibitory protein) did not survive beyond ~E11.5 (fig. S4), indicating that these proteins are dispensable for activation of caspase-8 by RIPK3 D161N.

To test whether RIPK3 D161N was deleterious in adult mice, we engineered *Ripk3*^{LSL/LSL} mice with *loxP*-Stop-*loxP* (LSL) transcription termination cassettes preventing expression of RIPK3 D161N during development (fig. S1B). This strategy was possible because *Ripk3*^{-/-} mice develop normally (6). We deleted the LSL cassettes and switched on expression of RIPK3 D161N in adults using ubiquitously expressed tamoxifen-inducible cre recombinase (CreERT2) (14). All *Ripk3*^{LSL/LSL} CreERT2 mice given tamoxifen developed diarrhea and lost between 13 and 28% of their body weight after 6 to 7 days, requiring that they be euthanized. No morbidity was observed in *Ripk3*^{+/-} or *Ripk3*^{LSL/+} mice expressing CreERT2 (Fig. 3A and fig. S5A). Histological analyses of *Ripk3*^{LSL/LSL} CreERT2 mice revealed degeneration of intestinal crypts, villous blunting and fusion, and mild enteritis (Fig. 3B). Lesions were mainly in the small intestine. Many mice had segmental, submucosal edema in the large intestine. Pyknotic and karyorrhectic lymphocytes in secondary lymphoid organs and thymic atrophy were consistent with lymphocyte death (fig. S5B). *Ripk3*^{LSL/LSL} CreERT2 mice showed no signs of morbidity after 5 days, but staining for cleaved caspase-3 revealed increased apoptosis in the small intestine (Fig. 3C). These results suggest that RIPK3 D161N affects caspase-8 in the small intestine like it does in the embryo vasculature.

We sought biochemical evidence of RIPK3 D161N affecting caspase-8 in *Ripk3*^{LSL/LSL} CreERT2 mouse embryo fibroblasts (MEFs) immortalized with adenovirus E1A. Culture with 4-hydroxytamoxifen, to promote expression of RIPK3 D161N, caused increased cell death that was not seen in *Ripk3*^{+/-} CreERT2 cultures (Fig. 3D). This death appeared to be apoptosis because it was blocked by the pan-caspase inhibitor zVAD.fmk (carbobenzoxycarbonyl-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone), but not by nec-1 (fig. S5C). As a control, nec-1 blocked the death of wild-type E1A-immortalized MEFs treated with TNF/zVAD.fmk (fig. S5D). Inhibition of apoptosis caused by RIPK3 D161N allowed us to isolate a complex containing FADD, caspase-8, RIPK1, and RIPK3 D161N (Fig. 3E). An equivalent complex was not recovered from similarly treated *Ripk3*^{+/-} CreERT2 cells. Further emphasizing the ability of RIPK3 D161N to promote apoptosis, the mutant RIPK3 protein was less abundant than was wild-type RIPK3. Poor expression of RIPK3 D161N did not result from decreased transcription of *Ripk3*^{D161N} mRNA (fig. S5E). In the absence of structural data for RIPK3 D161N, it is unclear why this catalytically inactive mutant engages RIPK1, FADD, and caspase-8 but wild-type RIPK3 does not. Regardless, our results indicate that the kinase activity of RIPK3 dictates whether a cell dies from necroptosis



or apoptosis. As such, small-molecule inhibitors of RIPK3 may have limited therapeutic benefit because of their potential to promote apoptotic cell death.

Nec-1 blocked necroptosis induced by TNF/zVAD.fmk (fig. S5C), was protective in an inflammatory disease model (15), and does not induce apoptotic cell death, suggesting that inhibition of RIPK1 rather than RIPK3 may have therapeutic benefit. To mimic RIPK1 inhibition in the whole animal, we generated *Ripk1* knock-in mice with Asp¹³⁸ of the HKD motif necessary for kinase activity mutated to Asn (fig. S6, A and B). Whereas *Ripk1*^{−/−} mice died soon after birth, *Ripk1*^{D138N/D138N} mice were viable. Consistent with a critical role for the kinase activity of RIPK1 in TNF-induced necroptosis, *Ripk1*^{D138N/D138N} bone marrow–derived macrophages (BMDMs) and E1A-immortalized MEFs were as resistant as *Ripk3*^{−/−} cells to killing by TNF/zVAD.fmk (Fig. 4A and fig. S6C). *Ripk1*^{D138N/D138N} cells expressed normal amounts of RIPK3 and MLKL (Fig. S6D). *Ripk1*^{D138N/D138N} mice also resembled *Ripk3*^{−/−} mice in their systemic response to TNF, exhibiting less hypothermia than did their wild-type counterparts (Fig. 4B). Unlike RIPK1 loss, RIPK1 D138N did not rescue embryonic lethality caused by RIPK3 D161N (fig. S4). This result is consistent with nec-1 not protecting MEFs ex-

pressing RIPK3 D161N (fig. S5B) and indicates that the kinase activity of RIPK1 is not required for activation of caspase-8 by RIPK3 D161N.

RIPK1 is required for TNF-induced nuclear factor κ B (NF- κ B) and mitogen-activated protein kinase signaling (16, 17). Wild-type RIPK1 and RIPK1 D138N restored these signals in *Ripk1*^{−/−} cells (18), suggesting that RIPK1 has an essential scaffold function in this setting, whereas its kinase activity is dispensable. Indeed, *Ripk1*^{D138N/D138N} and wild-type BMDMs were indistinguishable in their phosphorylation of inhibitor of NF- κ B α (I κ B α), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) in response to TNF (Fig. 4C). These results, together with the viability of *Ripk1*^{D138N/D138N} mice, are encouraging because they suggest that inhibiting the kinase activity of RIPK1 has no deleterious effects, at least in the short term. These *Ripk1*^{D138N/D138N} mice can be used to explore the contribution of RIPK1 and necroptosis to various mouse models of human disease.

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Supplementary Materials

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Highly Multiplexed Subcellular RNA Sequencing in Situ

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Understanding the spatial organization of gene expression with single-nucleotide resolution requires localizing the sequences of expressed RNA transcripts within a cell in situ. Here, we describe fluorescent in situ RNA sequencing (FISSEQ), in which stably cross-linked complementary DNA (cDNA) amplicons are sequenced within a biological sample. Using 30-base reads from 8102 genes in situ, we examined RNA expression and localization in human primary fibroblasts with a simulated wound-healing assay. FISSEQ is compatible with tissue sections and whole-mount embryos and reduces the limitations of optical resolution and noisy signals on single-molecule detection. Our platform enables massively parallel detection of genetic elements, including gene transcripts and molecular barcodes, and can be used to investigate cellular phenotype, gene regulation, and environment in situ.

The spatial organization of gene expression can be observed within a single cell, tissue, and organism, but the existing RNA localization methods are limited to a handful of genes per specimen, making it costly and laborious to localize RNA transcriptome-wide (1–3). We originally proposed fluorescent in situ sequencing (FISSEQ) in 2003 and subsequently developed methods to sequence DNA amplicons on a solid substrate for genome and transcriptome sequencing (4–7); however, sequencing the cellular RNA in situ for gene expression profiling re-

quires a spatially structured sequencing library and an imaging method capable of resolving the amplicons.

We report here the next generation of FISSEQ. To generate cDNA amplicons within the cell (fig. S1), RNA was reverse-transcribed in fixed cells with tagged random hexamers (fig. S2A). We incorporated aminoallyl deoxyuridine 5'-triphosphate (dUTP) during reverse transcription (RT) (fig. S2B) and refixed the cells using BS(PEG)9, an amine-reactive linker with a 4-nm spacer. The cDNA fragments were then circularized before rolling

circle amplification (RCA) (fig. S2C), and BS(PEG)9 was used to cross-link the RCA amplicons containing aminoallyl dUTP (fig. S2, D and E). We found that random hexamer-primed RT was inefficient (fig. S3A), but cDNA circularization was complete within hours (fig. S3, B to D). The result was single-stranded DNA nanoballs 200 to 400 nm in diameter (fig. S4A), consisting of numerous tandem repeats of the cDNA sequence. BS(PEG)9 reduced nonspecific probe binding (fig. S4B), and amplicons were highly fluorescent after probe hybridization (fig. S4C). As a result, the amplicons could be rehybridized many times, with minimal changes in their signal-to-noise ratio or position (fig. S4, D and E). Using SOLiD sequencing by ligation (fig. S5), the signal overlap over 27 consecutive sequencing reactions was ~600 nm in diameter (fig. S4F). In induced pluripotent stem (iPS) cells, the amplicons counterstained subcellular structures, such as the plasma membrane, the nuclear membrane, the nucleolus, and the chromatin (Fig. 1A, fig. S6, and movies S1 to S3). We were able to generate RNA sequencing libraries in different cell types, tissue

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sections, and whole-mount embryos for three-dimensional (3D) visualization that spanned multiple resolution scales (Fig. 1, B and C).

High numerical aperture and magnification are essential for imaging RNA molecules in single cells (8–10), but many gene expression patterns are most efficiently detected in a low-magnification and wide-field mode, where it typically becomes difficult to distinguish single molecules because of the optical diffraction limit and low sensitivity (11). To obtain a spot density that is high enough to yield statistically significant RNA localization, and yet sufficiently low for discerning individual molecules, we developed partition sequencing, in

which preextended sequencing primers are used to reduce the number of molecular sequencing reactions through random mismatches at the ligation site (Fig. 2A). Progressively longer sequencing primers result in exponential reduction of the observed density, and the sequencing primer can be changed during imaging to detect amplicon pools of different density.

Fluorescence microscopy can be accompanied by tissue-specific artifacts and autofluorescence, which impede accurate identification of objects. If objects are nucleic acids, however, discrete sequences, rather than the analog signal intensity, can be used to analyze the image. For FISSEQ,

putative nucleic acid sequences are determined for all pixels. The sequencing reads are then compared with reference sequences, and a null value is assigned to unaligned pixels. With a suitably long read length (L), a large number of unique sequences (n) can be used to identify transcripts or any other objects with a false-positive rate of approximately $n/4^L$ per pixel. Because the intensity threshold is not used, even faint objects are registered on the basis of their sequence, whereas background noise, autofluorescence, and debris are eliminated (Fig. 2B).

We applied these concepts to sequence the transcription start site of inducible mCherry mRNA in situ, analogous to 5' rapid amplification of cDNA ends–polymerase chain reaction (RACE-PCR) (12). After RT and molecular amplification of the 5' end followed by fluorescent probe hybridization (fig. S7A), we quantified the concentration- and time-dependent mCherry gene expression in situ (fig. S7B). Using sequencing-by-ligation, we then determined the identity of 15 contiguous bases from each amplicon in situ, corresponding to the transcription start site (fig. S7C). When the sequencing reads were mapped to the vector sequence, 7472 (98.7%) amplicons aligned to the positive strand of mCherry, and 3967 (52.4%) amplicons mapped within two bases of the predicted transcription start site (fig. S7D).

We then sequenced the transcriptome in human primary fibroblasts in situ (Fig. 3A) and generated sequencing reads of 27 bases with a median per-base error rate of 0.64% (fig. S8). Using an automated analysis pipeline (fig. S9), we identified 14,960 amplicons with size >5 pixels, representing 4171 genes, of which 13,558 (90.6%) amplicons mapped to the correct annotated strand (Fig. 3B, fig. S10, and table S1). We found that mRNA (43.6%) was relatively abundant even though random hexamers were used for RT (Fig. 3C). Ninety genes with the highest expression counts included fibroblast markers (13), such as fibronectin (*FNI*); collagens (*COL1A1*, *COL1A2*, *COL3A1*); matrix metalloproteinases and inhibitors (*MMP14*, *MMP2*, *TIMP1*); osteonectin (*SPARC*); stanniocalcin (*STC1*); and the bone morphogenesis–associated transforming growth factor (TGF)–induced protein (*TGFB1*), representing extracellular matrix, bone development, and skin development [Benjamini-Hochberg false discovery rate (FDR) $<10^{-19}$, 10^{-5} , and 10^{-3} , respectively] (Fig. 3D) (14). We made Illumina sequencing libraries to compare FISSEQ to RNA-seq. Pearson's r correlation coefficient between RNA-seq and FISSEQ ranged from 0.52 to 0.69 ($P < 10^{-16}$), excluding one outlier (*FNI*). For 854 genes with more than one observation, Pearson's r was 0.57 ($P < 10^{-16}$), 0.47 ($P < 10^{-16}$), and 0.23 ($P < 10^{-3}$) between FISSEQ and RNA-seq from fibroblasts, lymphocytes, and iPS cells, respectively (Fig. 3E). When FISSEQ was compared with gene expression arrays, Pearson's r was as high as 0.73 ($P < 10^{-16}$) among moderately expressed genes, whereas genes with low or high expression levels correlated poorly ($r < 0.4$) (fig. S11).

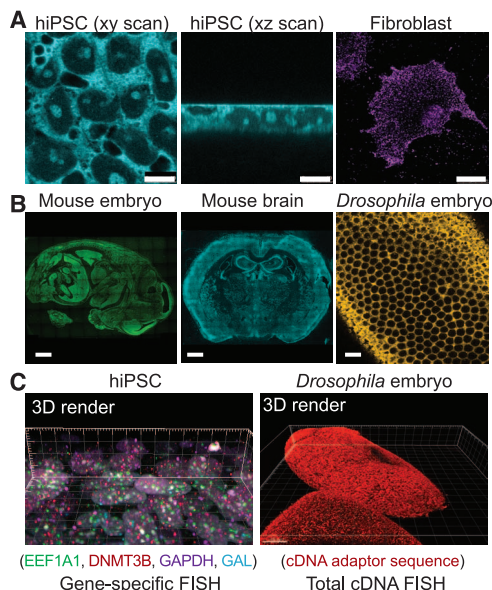


Fig. 1. Construction of 3D RNA-seq libraries in situ. After RT using random hexamers with an adapter sequence in fixed cells, the cDNA is amplified and cross-linked in situ. (A) A fluorescent probe is hybridized to the adapter sequence and imaged by confocal microscopy in human iPS cells (hiPSC) (scale bar: 10 μ m) and fibroblasts (scale bar: 25 μ m). (B) FISSEQ can localize the total RNA transcriptome in mouse embryo and adult brain sections (scale bar: 1 mm) and whole-mount *Drosophila* embryos (scale bar: 5 μ m), although we have not sequenced these samples. (C) 3D rendering of gene-specific or adapter-specific probes hybridized to cDNA amplicons. FISH, fluorescence in situ hybridization.

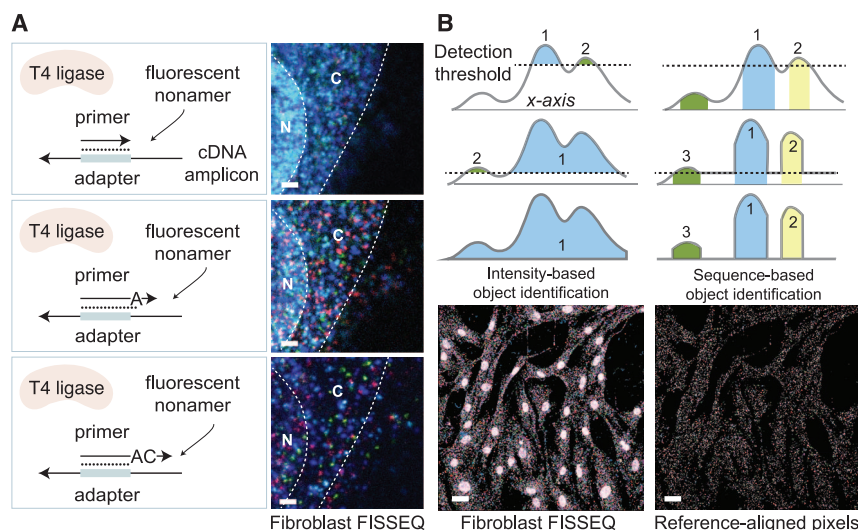


Fig. 2. Overcoming resolution limitations and enhancing the signal-to-noise ratio. (A) Ligation of fluorescent oligonucleotides occurs when the sequencing primer ends are perfectly complementary to the template. Extending sequencing primers by one or more bases, one can randomly sample amplicons at 1/4th, 1/16th, and 1/256th of the original density in fibroblasts (scale bar: 5 μ m). N, nucleus; C, cytoplasm. (B) Rather than using an arbitrary intensity threshold, color sequences at each pixel are used to identify objects. For sequences of L bases, the error rate is approximately $n/4^L$ per pixel, where n is the size of the reference. By removing unaligned pixels, the nuclear background noise is reduced in fibroblasts (scale bar: 20 μ m).

Highly abundant genes in RNA-seq and gene expression arrays were involved in translation and splicing (figs. S11 and S12), whereas such genes were underrepresented in FISSEQ. We examined 12,427 (83.1%) and 2533 (16.9%) amplicons in the cytoplasm and nuclei, respectively, and found

that nuclear RNA was 2.1 [95% confidence interval (CI) 1.9 to 2.3] times more likely to be non-coding ($P < 10^{-16}$), and antisense mRNA was 1.8 [95% CI 1.7 to 2.0] times more likely to be nuclear ($P < 10^{-16}$). We confirmed nuclear enrichment of *MALAT1* and *NEAT1* by comparing their

relative distribution against all RNAs (Fig. 3F) or mitochondrial 16S ribosomal RNA (rRNA) (table S2), whereas mRNA, such as *COL1A1*, *COL1A2*, and *THBS1*, localized to the cytoplasm (table S3). We also examined splicing junctions of *FN1*, given its high read coverage (481 reads over 8.9 kilobases).

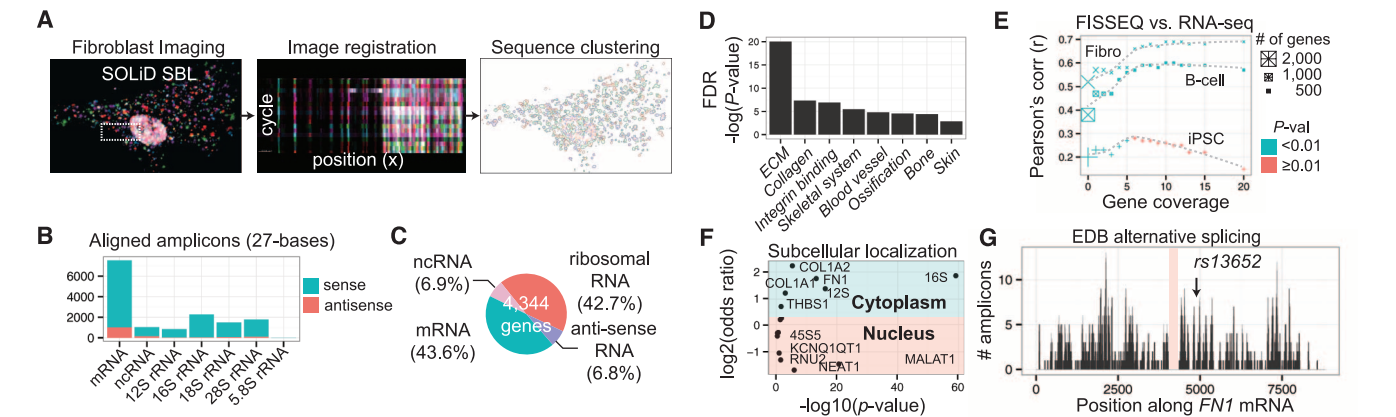
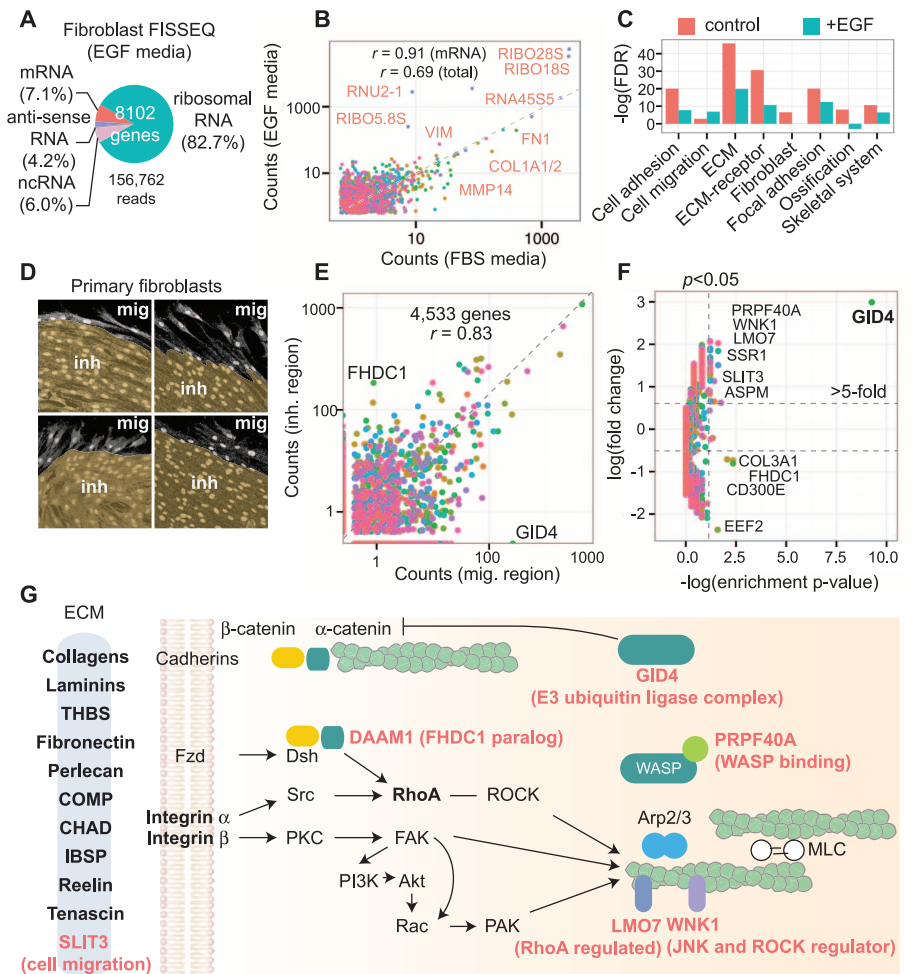


Fig. 3. Whole-transcriptome in situ RNA-seq in primary fibroblasts. (A) From deconvolved confocal images, 27-base reads are aligned to the reference, and alignments are spatially clustered into objects. (B) Of the amplicons, 90.6% align to the annotated (+) strand. (C) mRNA and non-coding RNA make up 43.6% and 6.9% of the amplicons, respectively. (D) GO term clustering for the top 90 ranked genes. (E) FISSEQ of 2710 genes

from fibroblasts compared with RNA-seq for fibroblast, B cell, and iPS cells. Pearson's correlation is plotted as a function of the gene expression level. (F) Subcellular localization enrichment compared to the whole transcriptome distribution. (G) Of the amplicons, 481 map to the *FN1* mRNA, showing an alternatively spliced transcript variant and a single-nucleotide polymorphism (arrow).

Fig. 4. Functional analysis of fibroblasts during simulated wound healing. (A) In EGF medium, rRNA makes up 82.7% of the amplicons. (B) EGF medium 147,610 reads compared with 13,045 reads from FBS medium (different colors denote genes). (C) The top 100 ranked genes from FBS versus EGF FISSEQ clustered for functional annotation. (D) An in vitro wound-healing assay allows cells to migrate (mig) into the wound gap. inh, contact-inhibited cells. The image segments are based on the cell morphology. (E) Comparison of 4533 genes from migrating and contact-inhibited cells. (F) Twelve genes are differentially expressed (Fisher's exact test $P < 0.05$ and >fivefold; 180 genes). (See table S4.) (G) The top 100 genes in fibroblasts are enriched for terms associated with ECM-receptor interaction and focal adhesion kinase complex (bold letters). During cell migration, genes involved in ECM-receptor-cytoskeleton signaling and remodeling are differentially expressed (red letters). THBS, thrombospondin; COMP, cartilage oligomeric matrix protein; CHAD, chondroaderhin; IBSP, integrin-binding sialoprotein; PKC, protein kinase C; FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase; MLC, myosin light chain; PAK, p21-activated protein kinase; WASP, Wiskott-Aldrich syndrome protein.



FNI has three variable domains referred to as EDA, EDB, and IIICS, which are alternatively spliced (15). We did not observe development-associated EDB, but observed adult tissue-associated EDA and IIICS (Fig. 3G).

We also sequenced primary fibroblasts in situ after simulating a response to injury, obtaining 156,762 reads (>5 pixels), representing 8102 annotated genes (Fig. 4A and fig S13, A to D). Pearson's r was 0.99 and 0.91 between different wound sites and growth conditions, respectively (Fig. 4B and fig. S13, E and F). In medium with epidermal growth factor (EGF), 82.7% of the amplicons were rRNA compared to 42.7% in fetal bovine serum (FBS) medium. When the 100 highest ranked genes were clustered, cells in FBS medium were enriched for fibroblast-associated GO terms, whereas rapidly dividing cells in EGF medium were less fibroblast-like (Fig. 4C) with alternative splicing of *FNI* (fig. S14). In regions containing migrating cells versus contact-inhibited cells, 12 genes showed differences in relative gene expression (Fisher's exact test $P < 0.05$ and >fivefold change) (Fig. 4, D to F, and table S4), eight of which were associated with the extracellular matrix (ECM)–receptor–cytoskeleton interaction, including *GID4*, *FHDC1*, *PRPF40A*, *LMO7*, and *WNK1* (Fig. 4G and table S4).

In summary, we present a platform for transcriptome-wide RNA sequencing in situ and demonstrate imaging and analytic approaches across multiple specimen types and spatial scales. FISSEQ correlates well with RNA-seq, except for genes involved in RNA and protein processing, possibly because some cellular structures or classes of RNA are less accessible to FISSEQ. It

is notable that FISSEQ generates far fewer reads than RNA-seq but predominantly detects genes characterizing cell type and function. If this finding can be generalized, FISSEQ may be used to identify cell types based on gene expression profiles in situ. Using partition sequencing to control the signal density, it may even be possible to combine transcriptome profiling and in situ mutation detection in a high-throughput manner (16–18). Using RNA barcodes from expression vectors, one can label up to 4^N (N = barcode length) cells uniquely, much more than is possible using a combination of fluorescent proteins (19). Similar to next-generation sequencing, we expect advances in read length, sequencing depth and coverage, and library preparation (i.e., fragmentation, rRNA depletion, targeted sequencing). Such advances may lead to improved stratification of diseased tissues in clinical medicine. Although more work remains, our present demonstration is an important first step toward a new era in biology and medicine.

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Supplementary Materials

www.sciencemag.org/content/343/6177/1360/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S14
Tables S1 to S4
References
Movies S1 to S6

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Structure of the Mitochondrial Translocator Protein in Complex with a Diagnostic Ligand

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The 18-kilodalton translocator protein TSPO is found in mitochondrial membranes and mediates the import of cholesterol and porphyrins into mitochondria. In line with the role of TSPO in mitochondrial function, TSPO ligands are used for a variety of diagnostic and therapeutic applications in animals and humans. We present the three-dimensional high-resolution structure of mammalian TSPO reconstituted in detergent micelles in complex with its high-affinity ligand PK11195. The TSPO-PK11195 structure is described by a tight bundle of five transmembrane α helices that form a hydrophobic pocket accepting PK11195. Ligand-induced stabilization of the structure of TSPO suggests a molecular mechanism for the stimulation of cholesterol transport into mitochondria.

The 18-kD translocator protein TSPO is preferentially expressed in mitochondrial membranes of steroidogenic tissues, and its gene family is present in almost all organisms (1–3). TSPO was first described as a peripheral benzodiazepine receptor, a secondary receptor for diazepam (1, 4). TSPO was subsequently found to be responsible for the transport of cho-

lesterol into mitochondria, thereby influencing the synthesis of neurosteroids (1, 5). TSPO also plays an important role in apoptosis (6) and stress adaptation (7). Expression of TSPO is strongly up-regulated in areas of brain injury and in neuro-inflammatory conditions including Alzheimer's and Parkinson's diseases (2). TSPO is located at contact sites between the outer and inner mitochon-

drial membrane, and was suggested to be part of (together with cyclophilin D, the voltage-dependent anion channel, and the adenine nucleotide translocator) the mitochondrial permeability transition pore (8).

TSPO ligands have potential diagnostic and therapeutic applications such as attenuation of cancer cell proliferation (6) and neuroprotective effects (2). TSPO ligands such as XBD-173 might also be useful in treating anxiety with reduced side effects relative to traditional benzodiazepine-related drugs (9). The best-characterized ligand of TSPO is 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), which binds to TSPO with nanomolar affinity in many species (10–13). PK11195 is used as a biomarker in positron emission tomography to visualize brain inflammation in patients with neuronal damage (2, 10). Moreover,

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the combination of TSPO ligands PK11195 and Ro5-4864 reduces levels of soluble β -amyloid in mice (14). The biochemical consequences of bind-

ing of PK11195 to TSPO include the stimulation of TSPO-mediated transport of cholesterol into mitochondria (12, 15).

Sequence analysis indicated that TSPO is organized into five transmembrane helices (1, 4, 16). Circular dichroism and nuclear magnetic resonance

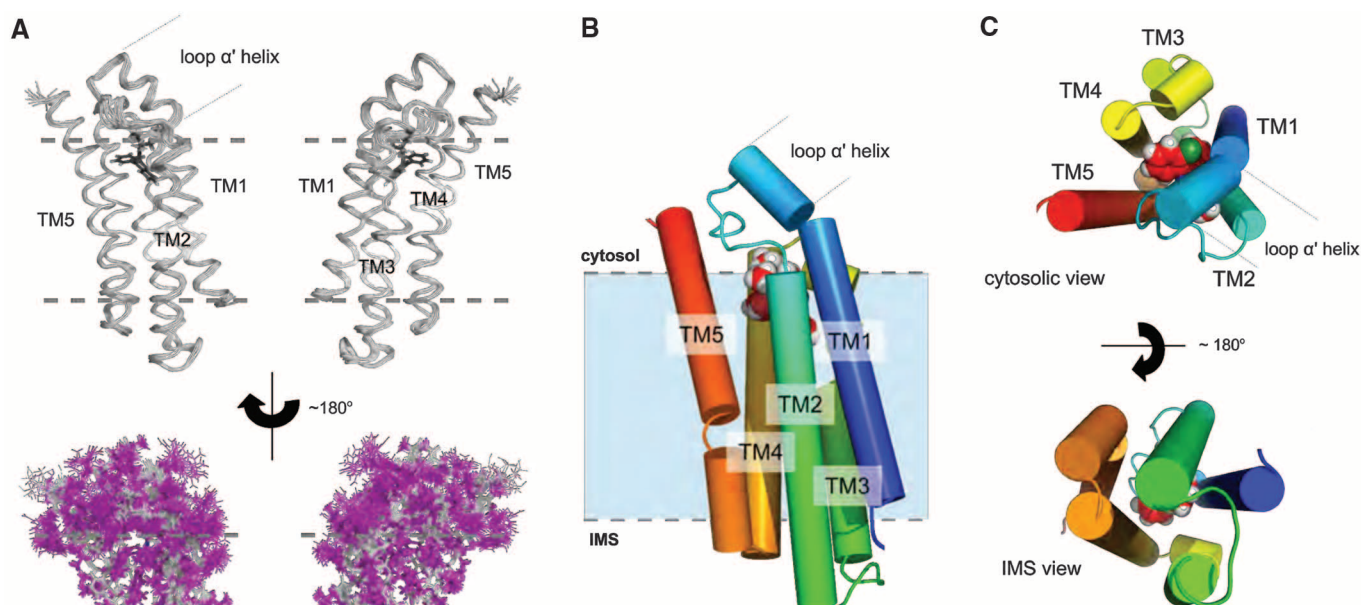


Fig. 1. High-resolution solution structure of the mTSPO-PK11195 complex. (A) Backbone ribbon trace (upper panel) and all-atom view (lower panel) of the 20 lowest-energy structures determined by NMR spectroscopy. The backbone and the side chains are shown in silver and magenta, respectively. The ligand is black. (B) Cylindrical representation of the lowest-energy complex structure, with the ligand atoms shown as spheres. (C) Cytosolic and IMS views of the mTSPO-PK11195 complex. Gray dotted lines in (A) and (B) indicate approximate membrane boundaries.

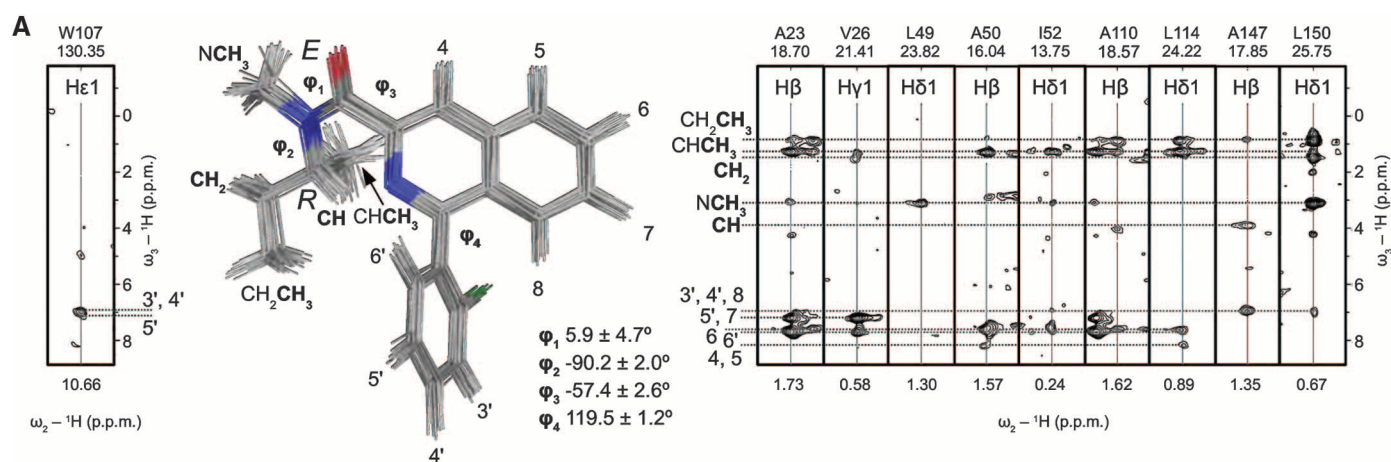


Fig. 2. Binding of PK11195 to TSPO. (A) Superposition of the 20 lowest-energy (R)-PK11195 conformations as found in the TSPO-PK11195 complex structure. Strips from 3D $\text{F1-}^{13}\text{C}$, ^{15}N -filtered/edited NOE spectroscopy (NOESY)- ^1H - ^{13}C heteronuclear single-quantum coherence (HSQC) (right) and 3D $\text{F1-}^{13}\text{C}$, ^{15}N -filtered/edited-NOESY- ^1H - ^{15}N -HSQC (left) experiments show intermolecular NOEs between PK11195 and selected TSPO residues. Ligand atoms are labeled. Dihedral angles ϕ_1 , ϕ_2 , ϕ_3 , and ϕ_4 are the average values found in the 20 lowest-energy structures $\pm$ SD. (B) Detailed view of the PK11195 binding cavity. Transmembrane helices are shown as green ribbons, the ligand in a stick representation. (C) TSPO residues that form the ligand-binding pocket. The same residues as in (A) are shown in a stick representation. Transmembrane helices are color-coded. Abbreviations: A, Ala; I, Ile; L, Leu; V, Val; W, Trp.

(NMR) studies on isolated peptides supported a predominantly helical structure of mouse TSPO (mTSPO) (17). Electron microscopy further provided a 10 Å resolution density map suggesting that a bacterial TSPO homolog—the tryptophan-rich sensory protein from *Rhodobacter sphaeroides*—folds into a five-helix bundle, which further associates into a homodimer (18). In addition, previous studies have shown that mammalian TSPO can be overexpressed and purified in a functional form (11). Drug ligands bind to the recombinant protein with a 1:1 stoichiometry and a pharmacological profile similar to that reported for the native protein (11, 12, 19).

We prepared recombinant mTSPO and reconstituted it into dodecylphosphocholine (DPC) micelles. In the absence of PK11195, NMR signals were highly overlapped and clustered into one region (fig. S1). The low signal dispersion might point to a dynamic nature of the free protein and explain difficulties in structural studies of this protein. However, in the presence of (*R*)-PK11195, which is used for positron emission tomography (10) and has a higher TSPO affinity than the racemate (20), high-quality NMR spectra were obtained (fig. S1). The combination of nuclear Overhauser enhancement (NOE) experiments and relaxation-optimized assignment

strategies together with complementary amino acid-specific labeling (21) allowed assignment of most resonances (figs. S2 and S3). Specifically, 98% of backbone and 95% of the side-chain resonances were assigned, including full stereospecific discrimination of all leucine and valine methyl groups. The completeness of the resonance assignment, together with the excellent quality of the NOE experiments at high magnetic fields, yielded more than 3300 NOE distance restraints and enabled direct detection of 61 TSPO-PK11195 contacts in three-dimensional (3D) F1-¹³C, ¹⁵N-filtered/edited NOE experiments (table S1 and figs. S3 and S4). The large number of medium- and long-range NOE contacts (fig. S4) defined the structure of residues Trp⁵ to Ser¹⁵⁹ of the TSPO-PK11195 complex at high resolution (Fig. 1, A and B), whereas the N- and C-terminal tails remained dynamic (fig. S5).

The 3D structure of the mTSPO-PK11195 complex comprises five transmembrane helices (TM1 to TM5) that tightly pack together in the clockwise order TM1-TM2-TM5-TM4-TM3 when viewed from the cytosol (Fig. 1, B and C). TM1 is perturbed by Pro¹⁵, whereas TM2 and TM3 are perturbed by the double-proline motifs Pro⁴⁴-Pro⁴⁵ and Pro⁹⁶-Pro⁹⁷, respectively. TM2 and TM3 also contain prolines at positions 51 and 81, respectively, and TM5 has a proline at position 139 that kinks it toward the intermembrane space (IMS). The C terminus is highly positively charged and exposed to the cytoplasm (fig. S6), as established by topological analysis (16). In contrast, equal numbers of positive and negative charges are present on the IMS side of the receptor (fig. S6). The charged patches exposed to the solvent might be important for interaction with proteins carrying endogenous TSPO ligands such as cholesterol (22). The loops between the transmembrane helices are short, with the exception of residues Gly²⁸ to Pro⁴⁵, which connect TM1 and TM2. The seven residues Glu²⁹ to Ala³⁵ fold into a short α helix (α') that is inclined with respect to the long axis of TM1 and positions the TM1-TM2 loop in close proximity to TM5 and the TM3-TM4 loop (Fig. 1). Because of the high sequence conservation of TSPO (fig. S7), the topology of the mTSPO structure is likely to be retained in other mammalian species as well as bacterial homologs.

TSPO binds drug ligands as a monomer but can also form dimers and higher-order oligomers (11, 23). The high quality of the NMR spectra suggested that mTSPO is predominantly monomeric when reconstituted into DPC micelles (figs. S1 and S2). To further support the monomeric state of the mTSPO-PK11195 complex, we performed titration experiments using the paramagnetic compound 16-doxyl-stearic acid. A continuous paramagnetic ring pattern around the protein molecule was observed (fig. S8), demonstrating that the central parts of all five transmembrane helices contact the hydrophobic tails of the detergent. In addition, the paramagnetic broadening data confirmed the topology of mTSPO and delineated the regions of TSPO that become

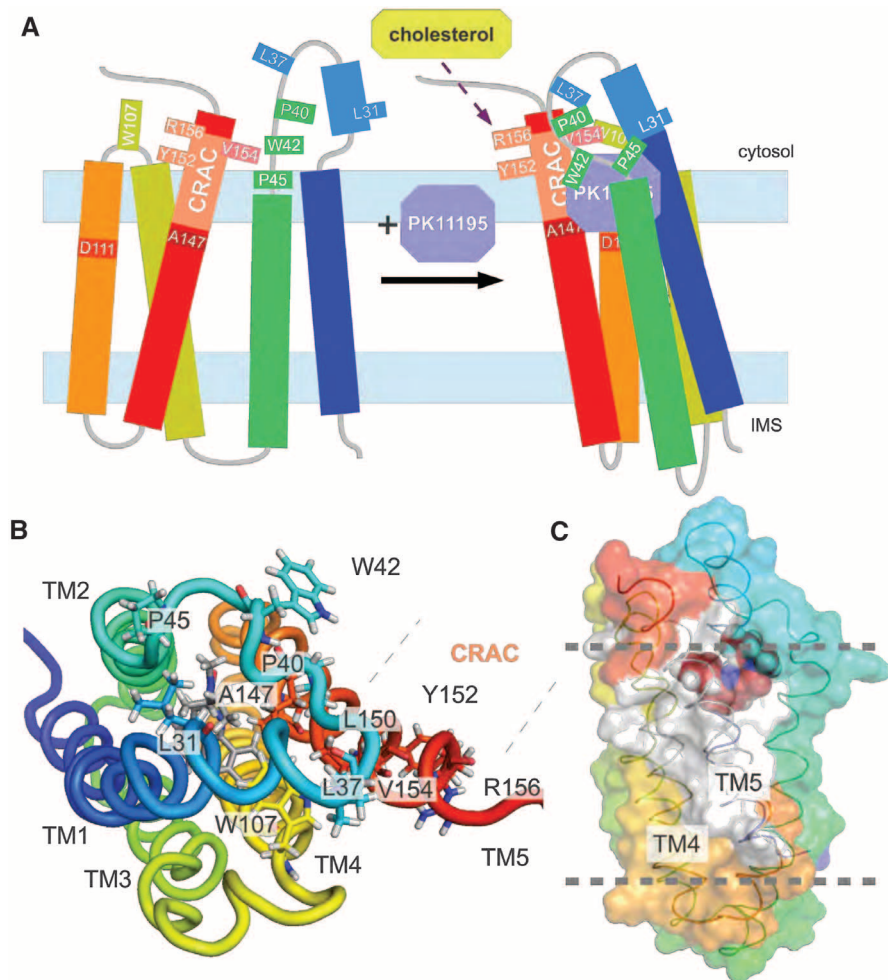


Fig. 3. Model for the ligand-induced modulation of the TSPO structure. (A) Cartoon representation of the conformation of TSPO in the absence (left) and presence (right) of PK11195. The representation of the ligand-bound state is based on the 3D structure of the mTSPO-PK11195 complex shown in Fig. 1. In agreement with circular dichroism (17), the secondary motifs in the ligand-free state have been assumed to be similar to the one in the mTSPO-PK11195 complex. The 2D correlation spectrum of mTSPO in DPC micelles displayed narrow signal dispersion (fig. S1), indicating increased mobility and decreased helix packing of the ligand-free structure. The cholesterol recognition amino acid consensus (CRAC) is labeled. Upon ligand binding, the cytosolic entrance to the channel is covered by the lid comprising the TM1-TM2 loop. (B) Atomic-resolution view onto the TM1-TM2 lid. The CRAC residues Tyr¹⁵² and Arg¹⁵⁶, which are essential for cholesterol binding (19, 27), are located on the outside of the TSPO structure and are not involved in PK11195 binding. (C) Surface representation of the TSPO-PK11195 complex highlighting the residues that were most protected after 28 hours of hydrogen-deuterium exchange (marked in white). The rest of the protein is colored as in (A). Gray dotted lines indicate the approximate membrane boundaries. Abbreviations: A, Ala; D, Asp; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

inserted into membranes (Fig. 1, B and C, and fig. S8).

In agreement with the 1:1 stoichiometry of the TSPO-PK11195 interaction (11), only a single set of protein-ligand contacts was observed (Fig. 2A and table S1). Sixty-one contacts of PK11195 to TSPO described a binding pocket formed by the five transmembrane helices in the upper cytosolic part of the helical bundle (Fig. 2, B and C). PK11195 binds to mTSPO with the *E*-amide rotamer (Fig. 2, A and B), although free in solution both the *E*- and *Z*-rotameric conformations are possible (24). PK11195 contacts several conserved residues in the binding pocket that is formed by residues Ala²³, Val²⁶, Leu⁴⁹, Ala⁵⁰, Ile⁵², Trp¹⁰⁷, Ala¹¹⁰, Leu¹¹⁴, Ala¹⁴⁷, and Leu¹⁵⁰ (Fig. 2, B and C, and fig. S7). One of these residues, Ala¹⁴⁷, is particularly interesting, as its natural polymorphism to threonine (Ala¹⁴⁷ → Thr polymorphism, rs6971) strongly affects TSPO binding of second-generation radioligands—but not PK11195—and thereby the application of these ligands in humans (25). The binding pocket is closed from the cytosolic side by the long loop between TM1 and TM2 (Fig. 1, A and B, and fig. S7). Deletion of residues 41 to 51 in mTSPO as well as site-directed mutagenesis in several species supported the importance of the TM1-TM2 loop for PK11195 binding (19, 26). The mode of PK11195-TSPO binding is likely to be important for other interactions, as PK11195 competes with several small molecules for binding to TSPO (1, 2, 10). In addition, synthetic or endogenous ligands might involve additional binding sites (1, 2, 10), providing a further level of regulation of TSPO function.

Cholesterol binds with nanomolar affinity to recombinant TSPO (11). The interaction occurs through the cholesterol recognition sequence (residues 147 to 159; Ala-Thr-Val-Leu-Asn-Tyr-Tyr-Val-Trp-Arg-Asp-Asn-Ser) at the C terminus of TM5 (Fig. 3A) (19, 27). The 3D structure of the TSPO-PK11195 complex reveals that the side chains of Tyr¹⁵², Tyr¹⁵³, and Arg¹⁵⁶, which are essential for cholesterol binding (19, 27), are located on the outside of the TSPO structure and point toward the membrane environment (Fig. 3B). They are therefore not involved in binding to PK11195, consistent with the finding that site-directed mutagenesis of these residues inhibited binding to cholesterol but not to PK11195 (19, 27). The location of residues essential for cholesterol binding at the outside of the TSPO structure, in combination with the known ability of cholesterol to dimerize, suggests that cholesterol binding can modulate the oligomerization of TSPO. Indeed, several transporters function as dimers (28). Residues from the cholesterol recognition sequence, as well as Leu¹¹² to Val¹¹⁵ in TM4, are highly stable, as evidenced by hydrogen-deuterium exchange (Fig. 3C and fig. S9). Thus, binding of PK11195 stabilizes the 3D structure of TSPO, in agreement with the pronounced increase in NMR signal dispersion upon addition of PK11195 (fig. S1). The ligand-induced stabilization of the TSPO structure might provide

a mechanism to promote transport of cholesterol (Fig. 3A) (12, 13), consistent with the observation that PK11195 markedly increased the binding of cholesterol to TSPO polymers (23).

The 3D structure of the TSPO-PK11195 complex reveals how the members of this important receptor family are organized at the molecular level and provides a basis for understanding the function of TSPO in physiological and pathological conditions.

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Supplementary Materials

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Figs. S1 to S9
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Epistasis and Allele Specificity in the Emergence of a Stable Polymorphism in *Escherichia coli*

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Ecological opportunities promote population divergence into coexisting lineages. However, the genetic mechanisms that enable new lineages to exploit these opportunities are poorly understood except in cases of single mutations. We examined how two *Escherichia coli* lineages diverged from their common ancestor at the outset of a long-term coexistence. By sequencing genomes and reconstructing the genetic history of one lineage, we showed that three mutations together were sufficient to produce the frequency-dependent fitness effects that allowed this lineage to invade and stably coexist with the other. These mutations all affected regulatory genes and collectively caused substantial metabolic changes. Moreover, the particular derived alleles were critical for the initial divergence and invasion, indicating that the establishment of this polymorphism depended on specific epistatic interactions.

Heritable differences in ecologically important traits can allow distinct lineages to arise and coexist. In sexual organisms, divergence typically occurs when populations are geographically separated, and the lineages may or may not persist in the event of later contact (1, 2). In asexual organisms, divergent lineages

can arise and persist even in sympatry if ecological opportunities are available (3–7). Divergent lineages have been seen to evolve in environments with unexploited resources (4, 6) or spatial gradients (3); these opportunities are sometimes generated by the organisms themselves through secretion of metabolites (5, 7) and other forms

of niche construction (8). Selective processes, including character displacement and trade-offs in life-history traits or metabolic functions, can promote divergence by causing negative frequency-dependent interactions between nascent lineages (1–7, 9). However, the genetic changes necessary to construct an ecologically distinct lineage are incompletely understood except when they involve single mutations (3, 5). Genome sequencing identified all of the mutations fixed during episodes of divergence in two earlier studies (10, 11) where multiple resources were exogenously provided, although the precise sets of mutations required for coexistence were not fully elucidated.

Here, we identify three mutations that together allow one bacterial lineage to invade and stably coexist with another (7, 12–14). Twelve populations were founded from the same strain of *Escherichia coli* and propagated in a glucose-limited minimal medium for >50,000 generations (15, 16). In the Ara-2 population, two lineages, S and L, diverged by 6500 generations and coexist thereafter (7, 12–14). Their coexistence involves niche construction through cross-feeding; the L ecotype grows faster on glucose but secretes by-products that S can better exploit, generating negative frequency-dependent selection. Global transcription profiling identified functional changes specific to the S lineage that appear to be important for its emergence and maintenance (14), but the mutations that caused those differences were not identified, nor were any specific differences shown to play a causal role. We sequenced the genomes of two clones sampled at 6500 generations from each lineage (17): 6.5S1, 6.5S2, 6.5L4, and 6.5L9. We identified candidate alleles and moved them between genetic backgrounds to investigate how the S type invaded and established a persistent lineage.

These clones average 199 mutations compared with the ancestor (18) (table S1); this population became hypermutable when a mutation affecting mismatch repair was fixed early in the experiment (19). Sixty-eight mutations were shared by all four clones (fig. S1 and table S2), implying that they occurred before the S and L lineages diverged. Fifty-five mutations were specific to the S clones and 36 to the L clones (tables S3 and S4). From these data, we estimate that the lineages split between generations 3500 and 4000 (17). However, the phenotypic differences that allowed coexistence arose later. Given hundreds of mutations, it was impossible to manipulate them all. Instead, we sought to identify a minimal set sufficient to allow the S type to invade and stably coexist with the L type. We reasoned that these conditions would require both S-specific mutations and beneficial mutations that arose before the S and L lineages diverged, by which time substantial fitness gains had already accrued (15).

We scrutinized the S- and L-specific mutations based on the lineage-specific differences in gene expression (14). Two S-specific mutations affected the *arcA* and *gntR* genes, which encode regulators of the TCA cycle and the Entner-

Doudoroff pathway, respectively (20, 21). We found both mutations in all S clones from 6500 generations onward, whereas at 6000 generations only the *arcA* allele was present, indicating that it arose before the *gntR* allele (Fig. 1A). We constructed a set of isogenic strains except for the *arcA* and *gntR* alleles in the ancestral and 6.5S1 backgrounds (17). We competed these strains against 6.5L4 using a reciprocal-invasion design (17) that allowed us to distinguish frequency-dependent and generic fitness effects. With both its derived *arcA* and *gntR* alleles, the 6.5S1 clone could invade 6.5L4, but it could not invade if either allele was replaced by the ancestral form (Fig. 1B and fig. S2A). Also, moving the *gntR* allele into a 6000-generation “pre-S” clone (6K3) that carried the *arcA* allele gave the ability to invade 6.5L4 (Fig. 1B and fig. S2B). Thus, the derived *arcA* and *gntR* alleles both contributed to establishing the S lineage.

When these *arcA* and *gntR* alleles were moved alone or together into the ancestral background, they did not allow invasion of 6.5L4 (Fig. 1C and fig. S3), although together they improved fitness by ~10% when rare (fig. S3), indicating ecological differentiation from the L lineage. However,

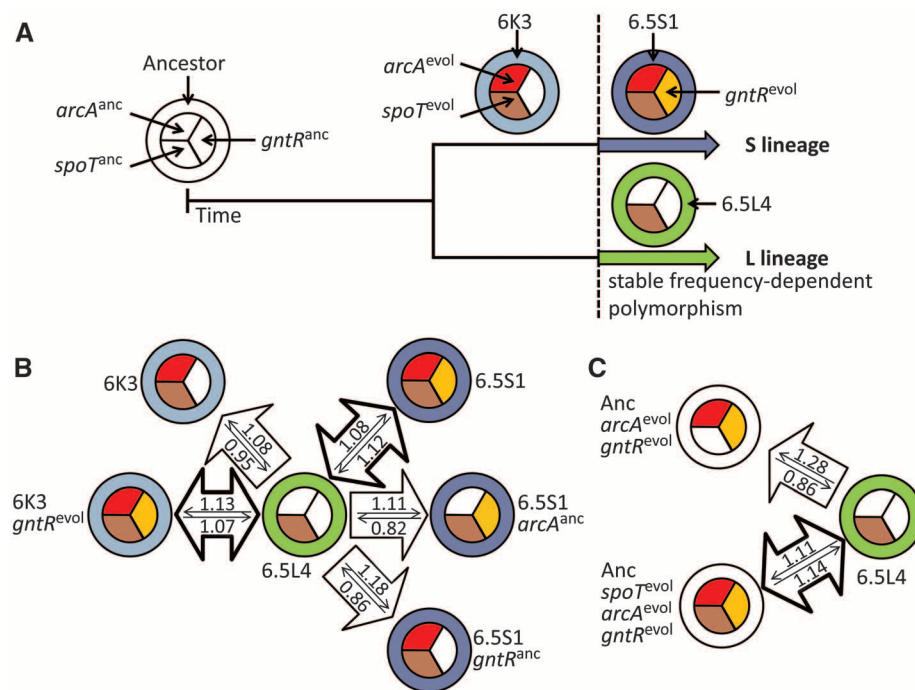


Fig. 1. Effects of focal mutations on ability of S to invade and coexist with L. (A) Genotypes are shown as circles, with outer rings denoting genetic backgrounds (white and colors for ancestral and evolved, respectively). The three sectors in each circle show the ancestral (anc, white) or experimentally evolved (evol, colors), i.e., derived, status for each of the *spoT*, *arcA*, and *gntR* genes. (B) Clones sampled at 6000 or 6500 generations and modified strains with different *arcA* or *gntR* alleles competed against clone 6.5L4. (C) Modified ancestral strains with derived *arcA* and *gntR* alleles and either ancestral or derived *spoT* allele competed against 6.5L4. Bidirectional arrows with thick edges indicate that each competitor, when initially rare, can invade the other, implying stable coexistence. Unidirectional arrows with thin edges point from superior competitors to losing strains, regardless of the initial ratio. Interior arrows point from initially rare (10% frequency) to initially common (90%) strains, and adjacent values show the fitness of rare relative to common competitors. Values >1 indicate that rare strains could invade common strains; values <1 indicate that rare strains could not invade. Means and 95% confidence intervals are shown in figs. S2 and S3.

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at least one more derived allele would be necessary to allow a constructed genotype to invade the L lineage. Mutations in *spoT*, a global regulatory gene, are among the most beneficial in the long-term populations (16, 22), and a mutation in *spoT* was fixed before the S and L lineages diverged (table S2). We combined the *spoT*, *arcA*, and *gntR* alleles in the ancestral background, and together they conferred the ability to invade and stably coexist with 6.5L4 (Fig. 1C and fig. S3). Thus, these mutations provide sufficient overall

adaptation and ecological differentiation to allow a constructed S ecotype to coexist with the evolved L ecotype.

When strains containing all possible combinations of the three derived alleles in the ancestral background competed against 6.5L4, we observed complex epistatic interactions (Fig. 2 and fig. S3). The *spoT* mutation increased fitness in all backgrounds, although the magnitude of its benefit varied. The *arcA* mutation was neutral or beneficial, depending on context; the *gntR*

mutation was deleterious or beneficial. Thus, the ability of the S lineage to invade and coexist with the L lineage depended on both the ecological opportunity and synergistic interactions among these mutations.

We also analyzed the individual and joint effects of these three alleles in the ancestral background during competition against the ancestor (17). As expected, the derived *spoT* allele was highly beneficial in this context, but neither the *arcA* nor the *gntR* allele was beneficial alone, nor

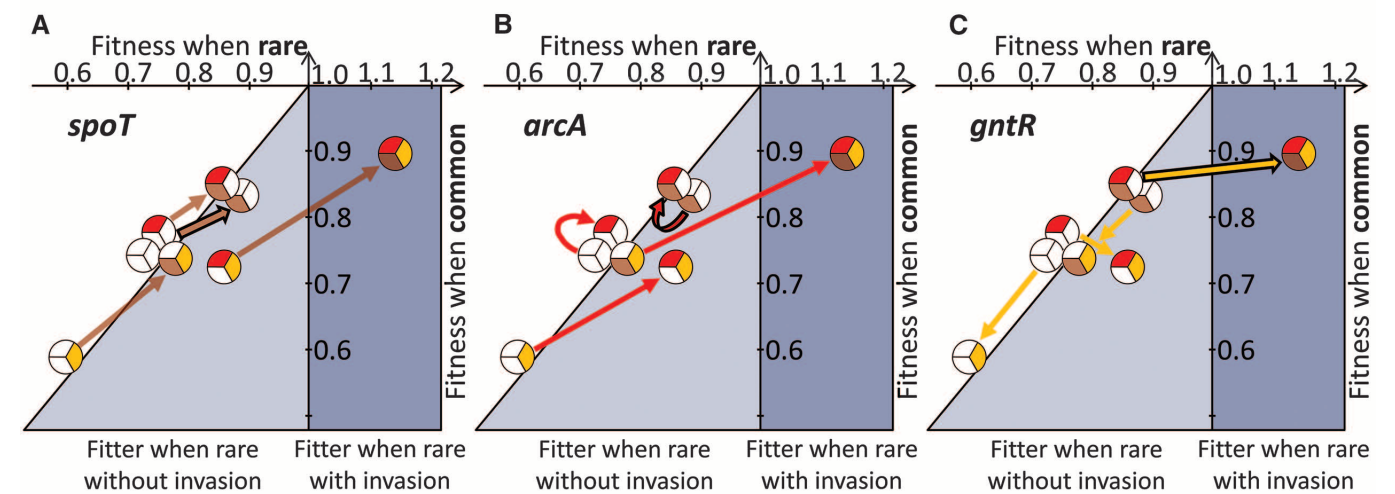


Fig. 2. Interactions among focal mutations. Each panel shows the fitness effects, when rare (*x* axis) and common (*y* axis), of adding the derived (A) *spoT*, (B) *arcA*, and (C) *gntR* allele to various backgrounds in competition with the 6.5L4 clone. Each allele is indicated by a colored sector, as in Fig. 1. The addition of an allele is shown as an arrow pointing away from the genetic background to which it was added. Arrows are colored according to the added

allele. Arrows with dark edges show events in the order they occurred during the evolution leading to the S lineage, although other mutations also occurred. The diagonal line represents frequency independence. A circle in the dark region on the right means the strain can invade when rare; circles in the lightly shaded region below the diagonal mean the strains are more fit when rare than when common but cannot invade.

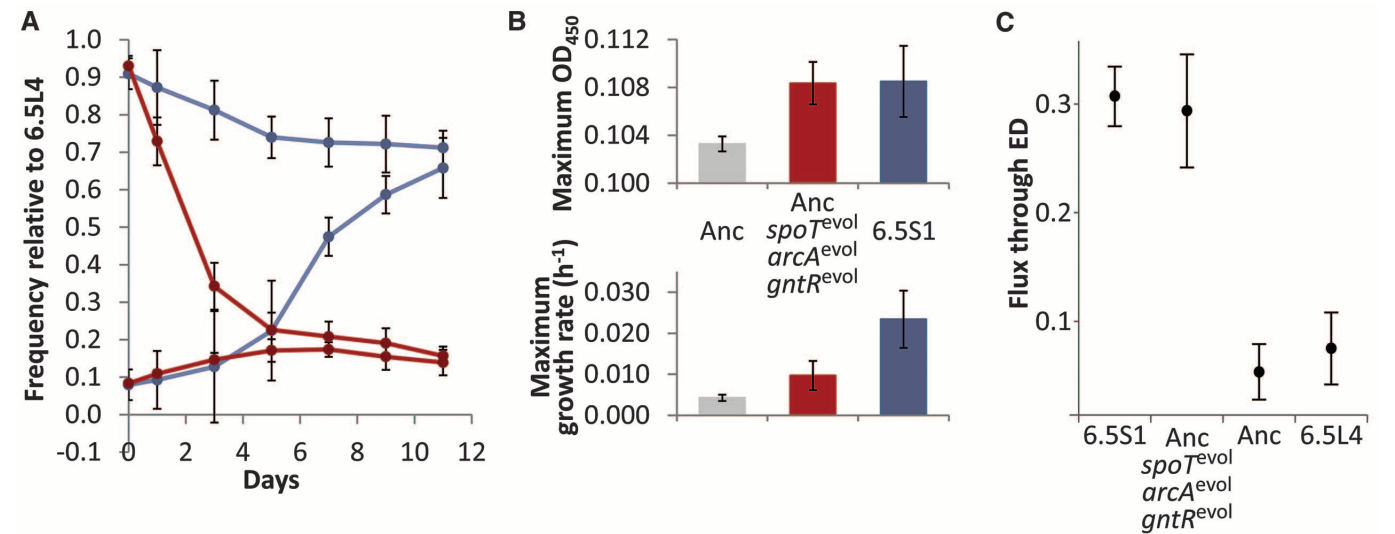


Fig. 3. Phenotypic comparisons of constructed S strain and clone 6.5S1. The constructed S ecotype has the derived *spoT*, *arcA*, and *gntR* alleles in the ancestral background. (A) Invasion and coexistence of the constructed S ecotype (red) and clone 6.5S1 (blue) with clone 6.5L4, starting from different initial frequencies. (B) Maximum optical density (OD₄₅₀) and growth

rate of the ancestral strain (gray), constructed S strain (red), and 6.5S1 (blue) in the supernatant obtained from a culture where 6.5L4 was previously grown. (C) Proportion of glucose flux through the Entner-Doudoroff (ED) pathway for ancestral, evolved, and constructed strains. Error bars in all panels are 95% confidence intervals.

did they increase fitness when combined sequentially with the *spoT* allele (fig. S4A). However, the derived *arcA* and *gntR* alleles were beneficial in the context of the S lineage; replacing either with its ancestral counterpart reduced fitness in competition with clone 6.5S1 (fig. S4B). Therefore, the establishment of the S lineage was a multistep process, with each step dependent on its ecological and genetic context.

We then analyzed the constructed S strain for other phenotypes that characterize the evolved S ecotype (17). The constructed S strain stably coexisted with 6.5L4, although with a lower equilibrium density than 6.5S1 (Fig. 3A). The constructed S type also grew faster and reached a higher density on L-conditioned supernatant (7) than the ancestor, although its growth rate was slower than 6.5S1 (Fig. 3B). These quantitative differences are unsurprising, however, because 6.5S1 harbors additional beneficial mutations not present in the constructed strain.

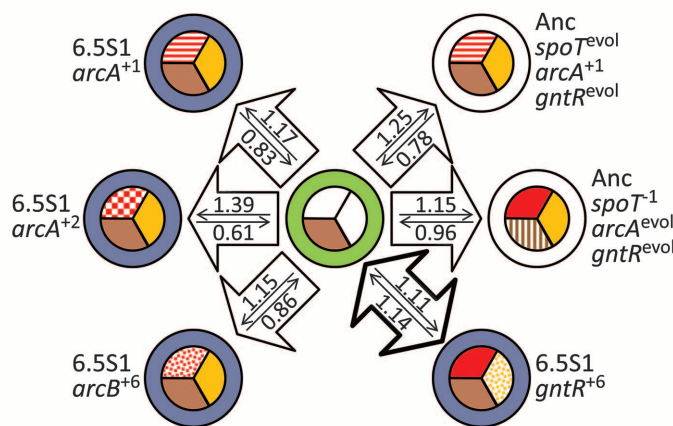
The constructed S strain also exhibited changes in central carbon metabolism (17), routing ~30% of catabolized glucose through the Entner-Doudoroff pathway rather than glycolysis (Fig. 3C). This flux is similar to that of 6.5S1 and significantly different from the ancestor (17). The derived *gntR* allele alone caused a large difference in flux pattern from the ancestor, whereas the *spoT* and *arcA* mutations had only small effects (fig. S5).

More subtle frequency-dependent interactions occur in some other replicate populations (23), and one generated an ecotypic polymorphism after evolving the ability to use citrate, an exogenously supplied resource (4, 10). However, Ara-2 appears unique in the strength of its cross-feeding interaction and the persistence of the polymorphism (7, 12–14, 23). However, many genes show parallel evolution (16): *spoT* mutations arose in seven other populations, *arcA* or *arcB* (encoding the regulator and sensor proteins of a two-component system) mutations in 10 others, and a *gntR* mutation in one other (table S6). In fact, one population, Ara+6, has mutations in all three genes.

To explore why Ara-2 followed its atypical evolutionary path, we replaced the *spoT*, *arcA*, and *gntR* alleles found in S with alleles from other populations (17) and examined how they affected the ability to invade and coexist with 6.5L4 (Fig. 4 and figs. S6 and S7). With so many alternative alleles, it was not possible to test them all. However, we produced six strains in the constructed S and 6.5S1 backgrounds, where one of the three alleles from S was replaced by an alternative allele from another population. In only one case, the *gntR* allele from Ara-6, did the alternative allele allow invasion of the L ecotype. With a *spoT* allele from Ara-1, the construct could not invade when rare; with four *arcA* and *arcB* replacements, the frequency-dependent interaction was lost and the constructs were less fit than with the S-derived *arcA* allele. Thus, the ecological differentiation and establishment of the S lineage required specific alleles in two genes. These specific mutations were responsible for, in essence, one-half of the stable coexistence of the S and L ecotypes. Other mutations, not yet identified, led to L-specific traits, including the propensity to secrete metabolites, the inability to use the by-products, or both.

In sum, we showed that three mutations, with complex epistatic and frequency-dependent effects, are sufficient to establish one partner in a strong and persistent ecological interaction. All three mutations affect regulators of gene expression, and regulatory changes are important for microbial evolution (22, 24). Other replicate populations fixed mutations in the same genes without evolving such persistent polymorphisms, implying that specific mutations produced qualitatively different evolutionary dynamics. Indeed, allelic exchanges confirm that alternative mutations in these genes preclude coexistence. Thus, a given gene may acquire many potentially beneficial mutations (16, 22, 25), but subtle variations may determine whether an evolving population remains monotypic or splits into stably coexisting lineages.

Fig. 4. Effects of alternative alleles on ability of S ecotype to invade and coexist with L ecotype. The *spoT* and *arcA* alleles in the constructed S strain were replaced by alternative alleles from populations Ara-1 and Ara+1, respectively. The *arcA* allele in clone 6.5S1 was replaced by alternative alleles from Ara+1 and Ara+2 or by a derived *arcB* allele from Ara+6. The *gntR* allele in 6.5S1 was replaced by an alternative allele from Ara+6. Color schemes for Ara-2–derived alleles and genetic backgrounds, arrows, and fitness values are as in Fig. 1. Alternative alleles from other populations have the same colors as the corresponding Ara-2 alleles, but with hatched or speckled motifs in the corresponding sectors. Means and 95% confidence intervals for each competing pair, along with controls, are shown in fig. S6.



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Supplementary Materials

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Materials and Methods
Figs. S1 to S7
Tables S1 to S6
Pipeline Code
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Humans Can Discriminate More than 1 Trillion Olfactory Stimuli

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Humans can discriminate several million different colors and almost half a million different tones, but the number of discriminable olfactory stimuli remains unknown. The lay and scientific literature typically claims that humans can discriminate 10,000 odors, but this number has never been empirically validated. We determined the resolution of the human sense of smell by testing the capacity of humans to discriminate odor mixtures with varying numbers of shared components. On the basis of the results of psychophysical testing, we calculated that humans can discriminate at least 1 trillion olfactory stimuli. This is far more than previous estimates of distinguishable olfactory stimuli. It demonstrates that the human olfactory system, with its hundreds of different olfactory receptors, far outperforms the other senses in the number of physically different stimuli it can discriminate.

To determine how many stimuli can be discriminated, one must know the range and resolution of the sensory system. Color stimuli vary in wavelength and intensity. Tones vary in frequency and loudness. We can therefore determine the resolution of these modalities along those axes and then calculate the number of discriminable tones and colors from the range

and resolution. Humans can detect light with a wavelength between 390 and 700 nm and tones in the frequency range between 20 and 20,000 Hz. Working within this range, researchers carried out psychophysical experiments with color or tone discrimination tasks in order to estimate the average resolution of the visual and auditory systems. From these experiments, they estimated that humans can distinguish between 2.3 million and 7.5 million colors (1, 2) and ~340,000 tones (3). In the olfactory system, it is more difficult to estimate the range and resolution because the dimensions and physical boundaries of the olfactory stimulus space are not known. Further, olfactory stimuli are typically mixtures of odor molecules that differ in their components. Therefore, the strategies used for other sensory modal-

ities cannot be applied to the human olfactory system. In the absence of a straightforward empirical approach, theoretical considerations have been used to estimate the number of discriminable olfactory stimuli. An influential study from 1927 posited four elementary odor sensations with sufficient resolution along those four dimensions to allow humans to rate each elementary sensation on a nine-point scale (4). The number of discriminable olfactory sensations was therefore estimated to be 9^4 or 6561 (4). This number was later rounded up to 10,000 and is widely cited in lay and scientific publications (5–7). Although this number was initially calculated to reflect how many olfactory stimuli humans can discriminate, it has also sometimes been used as the number of different odor molecules that exist, or the number of odor molecules that humans can detect. We carried out mixture discrimination testing to determine a lower limit of the number of olfactory stimuli that humans can discriminate.

Natural olfactory stimuli are almost always mixtures of large numbers of diverse components at different ratios. The characteristic scent of a rose, for example, is produced by a mixture of 275 components (8), although typically, only a small percentage of components contribute to the perceived smell. We reduced the complexity by investigating only mixtures of 10, 20, or 30 components drawn from a collection of 128 odorous molecules (table S1). These 128 molecules were previously intensity-matched by Sobel and co-workers, which enabled us to produce mixtures in which each component contributes equally to the overall smell of the mix-

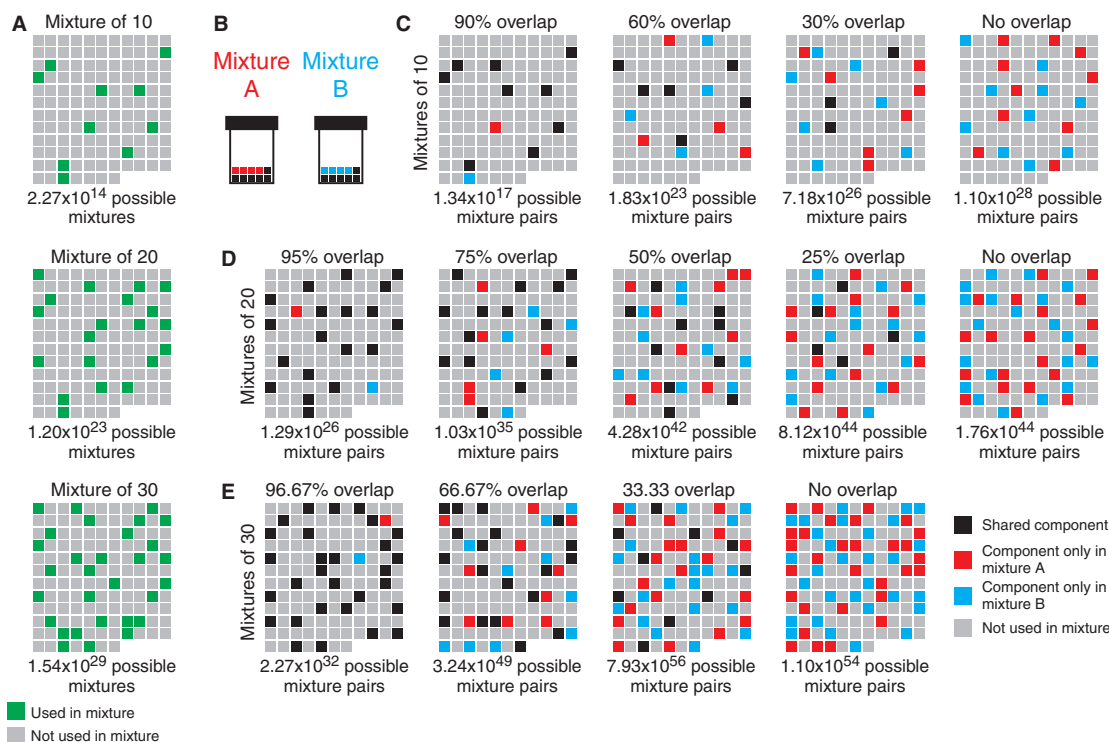


Fig. 1. Odor mixtures used to test the resolution of the human olfactory system. (A) Illustration of sample mixtures with exactly 10, 20, or 30 components (green squares) picked from a collection of 128 odorous molecules (gray squares)

and the number of possible mixtures of each type. (B) Example of one mixture pair. (C to E) Schematics of each of the 13 types of odor pairs used for discrimination tests, along with the total number of possible mixture pairs of each type.

ture (9). The 128 molecules cover much of the perceptual and physicochemical diversity of odorous molecules (10–12) because the collection contains most of a collection of 86 odorous molecules that were selected to be well distributed in both perceptual and physicochemical stimulus space (9).

To generate each mixture, we combined these components together at equal ratios. The 128 components can be combined into 2.27×10^{14} different mixtures of exactly 10, 1.20×10^{23} different mixtures of exactly 20, and 1.54×10^{29} different mixtures of exactly 30 (Fig. 1A). The most salient difference between two mixtures with the same number of components is the percentage of components in which they differ. We therefore performed psychophysical testing to determine the resolution of the human olfactory system along this axis. We asked by what percentage two mixtures must differ on average so that they can be discriminated by the average human nose. This percentage difference in components is the resolution of the olfactory system.

Subjects performed forced-choice discrimination tests to determine the discriminability of pairs of mixtures (referred to here as “mixture A” and “mixture B”) that varied in the percentage of shared components (Fig. 1B). In double-blind experiments, subjects were presented with three odor vials, two

of which contained the same mixture, whereas the third contained a different mixture. The testing procedure was computerized by using a custom-written application in which subjects were instructed to identify the odd odor vial on the basis of odor quality. Each subject completed the same 264 discrimination tests. The order of the tests was randomized. For each of 13 types of stimulus pairs (Figs. 1, C to E, and 2A), 20 different stimuli pairs were tested, for a total of 260 mixture discrimination tests. In addition, four control discrimination tests comprising individual odor molecules were interleaved across the mixture discrimination tests so as to measure general olfactory acuity and subject compliance. Because we wished to ensure that discrimination was based on odor quality differences and not small intensity differences, one of the three stimuli in a test was diluted in propylene glycol at a 1:2 ratio, the other at a 1:4 ratio, and the third was not diluted. The dilutions were assigned to the stimuli at random. The components and dilutions of all stimuli used in this study are given in table S2.

Twenty-eight subjects completed the study, but two were excluded from the analysis because they failed to correctly identify the odd odor in at least three of the four control tests. Data from 26 subjects [17 female; median age 30 (range of 20 to 48); 14 Caucasian, 5 African-American, 5

Asian, 2 Other; 4 Hispanic] are included in the analysis presented here.

Pairs of mixtures are more difficult to distinguish the more they overlap. At least half of the tested subjects could discriminate mixture pairs that overlapped by less than 75% of their components. Some could also discriminate mixture pairs that overlapped by 75 and 90%, but none could discriminate mixture pairs with more than 90% overlap (Fig. 2B). In this evaluation, the results of 20 mixture pairs of a certain type (for example, mixtures of 20 components that overlap by 75%) are pooled. Specific mixture interactions that are known to occur in odor mixtures, such as synergy and masking, are averaged out. To assess whether this biased our results, we also analyzed the results for each individual mixture pair, averaged across the 26 subjects (Fig. 2C). The results of this analysis were very similar. Of the 260 pairs of mixtures that were tested for discriminability, subjects performed above chance level for 227. For 148 of those, 14 or more of the 26 subjects (54%) chose the correct vial, resulting in a statistically significant difference from chance (Fig. 2C).

The resolution (or difference limen) of the visual and auditory system is defined as the difference in frequency between two stimuli that is required for reliable discrimination. In the olfactory system, resolution can be defined as the highest percentage overlap in components between two mixtures at which those mixtures can be distinguished. The resolution of the olfactory system is not uniform across the olfactory stimulus space. For example, half of the mixtures of 20 components with 50% overlap could be discriminated, whereas the other half were indistinguishable (Fig. 2C, middle). Non-uniform resolution across stimulus spaces is also found in other sensory systems. In hearing, for example, frequency resolution is much better at low than at high frequencies (3). In vision, wavelength discrimination is best near 560 nm, at which a difference in wavelength of 0.2 nm can be discriminated under optimal conditions (13).

We extrapolated functions that relate the percentage mixture overlap to mixture discriminability from our data. The function that relates the percentage mixture overlap (x) with the percentage of subjects that can discriminate the mixtures (y) is $y = -0.81x + 91.45$ (Fig. 3A). The function that relates the percentage mixture overlap (x) with the percentage of discriminable pairs (y) is $y = -0.77x + 94.22$ (Fig. 3B). According to these formulae, most subjects can discriminate mixture pairs that overlap by less than 51.17%. Most pairs that overlap by less than 57.43% can be discriminated.

To calculate a lower limit of how many discriminable mixtures there are, the number of possible mixtures and the difference between two mixtures that renders them indistinguishable have to be known. There are 2.27×10^{14} possible mixtures of exactly 10 (out of 128), 1.20×10^{23} possible mixtures of exactly 20, and 1.54×10^{29} possible mixtures of exactly 30. Using the numbers from our data, a lower limit of how many discriminable mixtures there are can be calculated. Mathematically, this

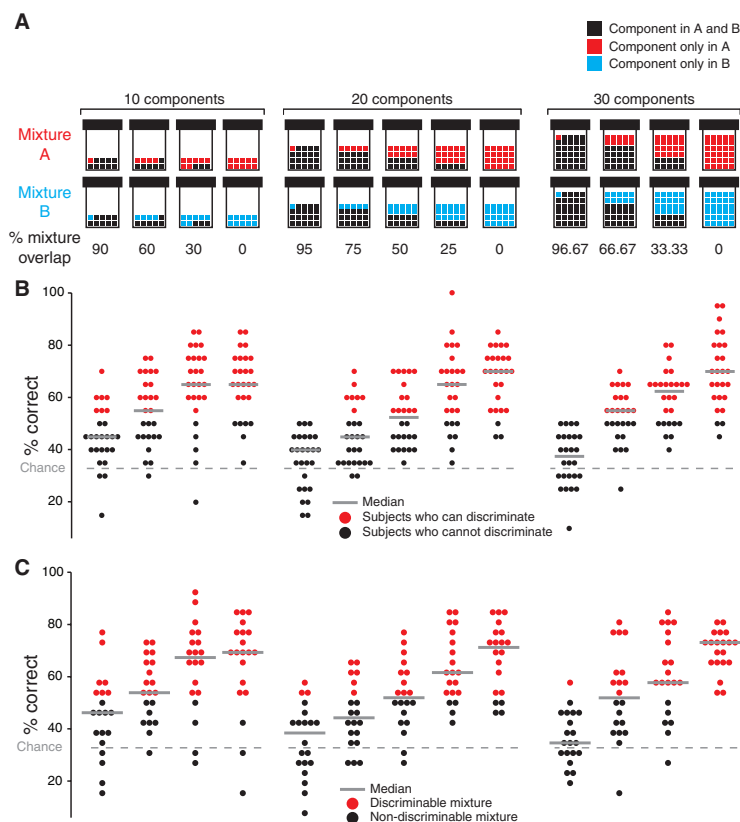


Fig. 2. An empirical investigation of the resolution of the human olfactory system. (A) Schematic of the discrimination tests carried out for mixtures of 10, 20, or 30 odor molecules. (B and C) Results of discrimination tests with 26 subjects asked to discriminate mixtures of 10 (left), 20 (middle), or 30 (right) components with decreasing overlap from left to right. The dotted line represents the chance detection level (33.3%). For (B), dots represent performance of individual subjects across 20 mixture pairs. For (C), dots represent average performance of all 26 subjects for a given mixture pair. Statistically significant discriminability (red dots) was assessed with a χ^2 test; $P < 0.05$.

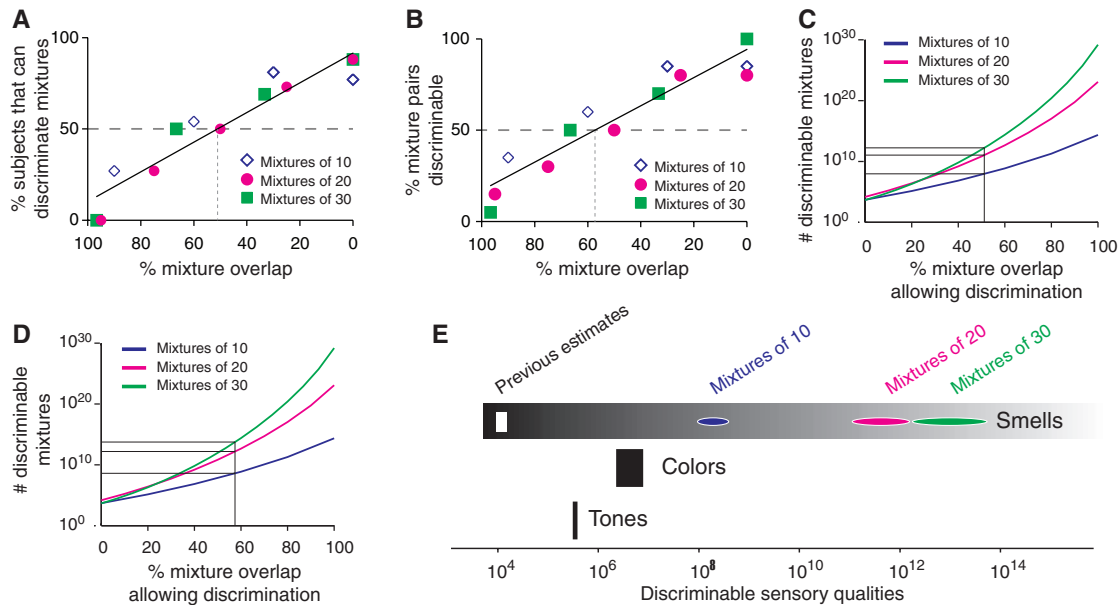


Fig. 3. The number of discriminable olfactory stimuli. (A) Discrimination capacity of subjects according to percentage of mixture overlap. (B) Discriminability of mixture pairs according to percentage of mixture overlap. (C) Extrapolation of the number of discriminable mixtures derived from (A). (D)

Extrapolation of the number of discriminable mixtures derived from (B). (E) Summary of discriminable sensory stimuli across sensory modalities. Data are curated from the following sources: smells (previous estimates) (5–7), tones (3), and colors (1, 2).

presents a coding problem that can be formulated in information theory as a problem of packing spheres in multidimensional space (14). The solution to this problem (details are available in the supplementary materials, materials and methods) shows that a resolution of 51.17% overlap results in 9.37×10^7 distinguishable mixtures of exactly 10, 1.12×10^{11} mixtures of exactly 20, and 1.72×10^{12} mixtures of exactly 30 (Fig. 3C). A resolution of 57.43% overlap results in 4.01×10^8 , 1.55×10^{12} , and 5.58×10^{13} discriminable mixtures, respectively (Fig. 3D).

Mixtures that overlap by less than 51.17% can be discriminated by the majority of subjects, which means that humans can, on average, discriminate more than 1 trillion mixtures of 30 components. However, there are large differences between subjects. The number of discriminable mixtures with 30 components in one subject of this study is 1.03×10^{28} , whereas it is only 7.84×10^7 in another subject (fig. S1).

One can calculate the number of mixtures that can be discriminated by either using the discrimination capacity of subjects or by the discriminability of stimulus pairs. Depending on what criteria are used, our results show that humans can discriminate 1.72×10^{12} or 5.58×10^{13} mixtures of 30 components out of the collection of 128 odorous molecules. 1.72×10^{12} may seem like an astonishingly large number. However, there are 1.54×10^{29} possible mixtures of 30 from the 128 components used here. Therefore, if there are 1.72×10^{12} discriminable stimuli, this means that for each mixture tested there will be 8.95×10^{16} other mixtures that cannot be discriminated from it.

Our results show that there are several orders of magnitude more discriminable olfactory stimuli than colors (1, 2) or tones (3) (Fig. 3E). Colors are spatially arranged to create a large number of visual objects that are the building blocks of

visual experiences, and tones can be combined to form a large number of chords that form auditory objects. The number of visual and auditory objects is much larger than the number of colors and tones, but it is unknown how many of these objects humans can discriminate. However, the difference between the number of discriminable olfactory stimuli and colors or tones is even larger if one considers that the number of discernible tones and colors includes stimuli that differ in loudness or brightness. We focused here only on stimulus quality and ruled out intensity-based discrimination. Thus, our estimate of 1.72×10^{12} is a conservative one yet is still several orders of magnitude higher than previous estimates of the number of discriminable olfactory stimuli (5–7). The actual number of distinguishable olfactory stimuli is likely to be even higher than 1.72×10^{12} for three reasons. First, it is currently not known how many odorous molecules there are or how many of them can be discriminated from the others. However, there are considerably more possible odorous molecules than the 128 different components that we used. Second, components can be combined in mixtures of more than 30 components. Third, even mixtures with the same components can be distinguished if the components are mixed at different ratios (15). Our results therefore establish only a lower limit of the number of discriminable olfactory stimuli. Although this lower limit of greater than 1 trillion is several orders of magnitude more than distinguishable colors or tones, it is presumably dramatically lower than the actual number of discriminable olfactory stimuli.

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Supplementary Materials

www.sciencemag.org/content/343/6177/1370/suppl/DC1
Materials and Methods
Fig. S1
Tables S1 and S2

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Control Profiles of Complex Networks

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Studying the control properties of complex networks provides insight into how designers and engineers can influence these systems to achieve a desired behavior. Topology of a network has been shown to strongly correlate with certain control properties; here we uncover the fundamental structures that explain the basis of this correlation. We develop the control profile, a statistic that quantifies the different proportions of control-inducing structures present in a network. We find that standard random network models do not reproduce the kinds of control profiles that are observed in real-world networks. The profiles of real networks form three well-defined clusters that provide insight into the high-level organization and function of complex systems.

Many natural complex systems are important or vital to human life, society, and governance. Undesirable behavior of such systems, observed in the form of disease, epidemics, economic collapse, and social unrest, has generated considerable interest in being able to control complex systems modeled as networks. Previous work has already established the connection between control theory and complex networks (1–3). Controlling a complex directed network reduces to (i) the selection of specific nodes that, by virtue of being directly controlled, can indirectly drive the rest of the network to any arbitrary state and (ii) the prescription of time-varying control inputs that, when applied to these nodes, will drive the system to a desired state.

Work to date has focused on the number of controls and their attachment points required by a complex network (4). Existing studies have shown how the mathematics of structural controllability can be used to identify a minimum number of controls that can drive the system as a whole to any arbitrary state. A main outcome of past work revealed that the degree distribution of a network alone (i.e., the probability distribution over the number of links that nodes have) is correlated with the minimum number of controls (1).

Understanding the control properties of a complex system requires not just knowing how many controls are needed, but also characterizing the functional origin of each control, an explanatory detail not provided by the degree distribution correlation. For example, a biochemical system and a financial system might require the same number of controls, but the structures within the networks that give rise to these controls could be quite different. Ultimately, planning and implementing a control scheme on a network will depend greatly on how these control points are situated in the network.

The formulation of control that we employ, structural controllability, formalizes the idea that, in the absence of a feedback loop (or, more generally, a cycle), a given node can control at most one of its immediate neighbors (5). Thus, the structure of the propagation of control influence through the network consists of a backbone of directed

paths, called stems, each driven by an independent control (6). These paths can then control cycles that are inherently self-regulatory (see supplementary text). However, ultimately it is these stems that dictate the need for controls: There must be one control for each stem in the system (7).

An immediate question, then, is how many stems exist in the system. The location of stems is largely dictated by two topological network features: sources, which are nodes with edges only pointing away from them (e.g., node A in Fig. 1), and sinks, which are nodes with edges only pointing toward them (e.g., nodes F and G in Fig. 1). A source, because it does not have edges entering it, can only appear at the origin of a stem and therefore must be directly controlled. Similarly, a sink, lacking edges to influence other nodes, must lie at the end of a stem. The number of stems in a complex system, then, is at least $\max(N_s, N_t)$, where N_s and N_t are the number of sources and sinks, respectively. Without lack of generality, we consider here a graph with no disconnected nodes (8).

In situations where a path must branch into two or more paths in order to reach all nodes, a dilation occurs: Additional stems must be introduced at these branching points, each one requiring a separate control (see supplementary

text). Some of these dilations may arise due to a surplus of sink nodes ($N_t > N_s$). In this case we call the dilations due to surplus sink nodes, $N_e = \max(0, N_t - N_s)$, external dilation points (see the branching at node E to F and G in Fig. 1). The remaining stem dilations give rise to a class of controls due to more nuanced network structures, called internal dilation points, N_i (see the branching at node A to nodes B and C in Fig. 1).

Taken together, the minimum number of independent controls (N_c) required for full control of a complex network is, then, the sum of the number of source nodes, external dilation points, and internal dilation points,

$$N_c = N_s + N_e + N_i \quad (1)$$

with N_s , N_e , and N_i not all zero (4, 9). One way to calculate N_c directly is by using a maximum matching algorithm, which, while accurate, is expensive for large networks (see supplementary text).

With this understanding, we can examine the breakdown of the origin of controls in terms of amounts of source nodes, external dilation points, and internal dilation points for different classes of real-world networks, as well as for widely used generative network models (see supplementary text for the complete listing). To make direct comparisons across networks of different size, in the following discussion we normalize by the number of nodes, N , i.e., $(n_s, n_e, n_i) = (N_s/N, N_e/N, N_i/N)$ and $n_c = N_c/N$.

For any network, $n_c^{sc} = n_s + n_e$ can be computed directly from the network's degree distribution, namely, the sum of the fraction of nodes with in-degree zero and out-degree zero. This explains why Liu *et al.* found that the degree distribution of a network correlates so strongly with the number of controls (1). Our findings show that knowing the full degree distribution is often unnecessary since, on average, the number of con-

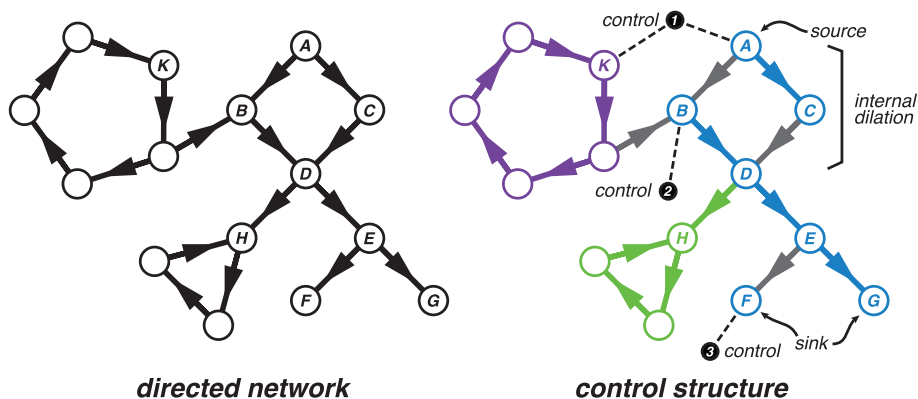


Fig. 1. An example of (left) a simple directed network and (right) one of the possible minimum control structures, constructed of stems (blue) and cycles (purple and green). There is one source (node A) and two sinks (nodes F and G). Three controls are required arising from one source (node A), one external dilation (due to two sink nodes F and G), and one internal dilation (due to the A-B-C-D topology). The dashed lines indicate how control signals enter the network. For control 1, the same signal is sent to nodes A and K. Controls 2 and 3 send different signals to nodes B and F, respectively. Gray arrows indicate edges that are not involved in the control structure of the network. See the supplementary text for additional details regarding the determination of the control structure and its various components.

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trols is dominated by only two points in the degree distribution: sources and sinks (i.e., nodes with in-degree = 0 or out-degree = 0, respectively).

Indeed, the fraction of nodes that are internal dilation points ($n_i = n_c - n_c^{se}$) is, in many cases, relatively small in both real networks and the random network models that are often used to approximate real-world networks. Across a total of 70 real networks—sampled from ecological, economic, neural, organizational, social, transcriptional, and technological systems (see table S2)— n_i averaged 14.0%. We observed low n_i across random networks as well: 3.0, 3.0, 2.8, and 24.9% in Erdos-Renyi (ER), Barabasi-Albert (BA), local attachment (LA), and duplication-divergence (DD) networks, respectively (10–13). When n_i is small, as is the case in many networks, the bound given by n_c^{se} is quite tight. Notably, DD networks exhibit a higher fraction of internal dilation points ($n_i = 24.9\%$), but still small enough to meaningfully employ the estimates above.

By simply counting source and sink nodes (a task that is linear in the number of nodes), we obtain a relatively good lower bound on the number of controls. This approach is a marked improvement over the maximum matching algorithm, which is used to obtain the exact number of minimum controls. This algorithm requires

computational steps proportional to the number of edges, which can be quadratically greater than the number of nodes (see supplementary text).

Moreover, this estimate performs as well as one based on the full degree distribution, despite requiring much less information. In Fig. 2, we compare the predictive power of using n_s and n_e alone (n_c^{se} , blue) against the number of controls implied by the network’s full degree distribution (n_c^{deg} , red). We find that, on average across all the surveyed networks, the additional information from the full degree distribution accounts for only 5.1% improvement in the prediction for real networks and 2.7% (ER), 0.6% (BA), 0.7% (LA), and 7.3% (DD) for synthetic networks (see fig. S1 for further details). This further indicates that additional statistics beyond the degree distribution will be needed to fully capture the number of internal dilations and to reduce these prediction errors.

Although prediction is a powerful use for our framework, we now return to the original question regarding the decomposition of controls in networks. To make an equitable comparison across networks with different numbers of controls, but potentially similar relative composition of controls, we modify Eq. 1 to represent the fractions of controls due to source nodes ($\eta_s = N_s/N_c$), external dilation points ($\eta_e = N_e/N_c$), and internal dilation points ($\eta_i = N_i/N_c$) by normalizing the equation with respect to the number of controls, N_c :

$$\eta_s + \eta_e + \eta_i = 1 \tag{2}$$

Because N_c can be computed by means of a maximum matching algorithm (see supplementary text), and N_s and N_e are obtained from analysis of node degrees, we can trivially solve for N_i . Our goal is no longer to estimate N_c ; hence, defining N_i as a function of N_s , N_e , and N_c is not circular be-

cause each of these terms can be directly computed. We obtain the control profile for a complex network, given by the triple (η_s, η_e, η_i) . Using these control profiles and their corresponding plots (see fig. S4), we can investigate the extent to which the profiles of networks differ.

We have already established that, of the synthetic networks considered, few have appreciable numbers of internal or external dilations. This is largely due to the way in which these networks are generated. The generative models that yielded networks with few internal dilations had one of two key features. BA and LA networks grow by new node attachment: At each time step, a new node is added that has no inbound neighbors. Preferential attachment further does not favor creating links to these new sources, yielding an enrichment in sources. ER, by contrast, creates sources and sinks with equal probability, making it difficult for generated networks to have substantial numbers of external dilations.

By definition, a dilation requires an expansion point in the network—a region where a smaller number of nodes link into a larger number of nodes. Preferential attachment is biased against expansion, favoring linking into smaller populations of high-degree nodes. ER also has no built-in bias toward expansion points. DD, however, does, by virtue of creating new nodes through copy events (14). Overall, this suggests that the expected values of a control profile may be anticipated and understood by appealing to the formation mechanism that generated it rather than particular high-level network properties that the mechanism induces.

Real networks have control profiles that are vastly different from the synthetic networks considered (see Fig. 3). This is related to the observation above—that existing generation methods are biased toward source-based controls. How-

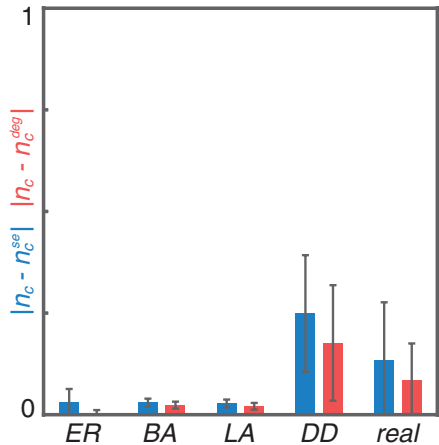


Fig. 2. Source and sink nodes dominate the correlation between degree distribution and the number of required controls. The error in the approximation of the fraction of required controls is only slightly improved by incorporating the nonzero terms of the degree distribution ($n_c^{deg} = N_c^{deg}/N$ is the fraction of required controls after a degree-preserving shuffle; $n_c^{se} = n_s + n_e$ is the fraction of source and sink nodes in the network). The mean improvement in approximation error for synthetic networks—Erdos-Renyi (ER), Barabasi-Albert (BA), local attachment (LA), and duplication-divergence (DD)—and real networks is indicated by the difference in bar heights. Synthetic networks were generated from a comprehensive set of parameters with network sizes, N , ranging from 100 to 50,000; average degree, L/N , ranging from 2 to 58; and for DD networks, a probability to retain copied edges from 0.1 to 0.9 (see supplementary text). The error bars represent one standard deviation from the mean approximation error.

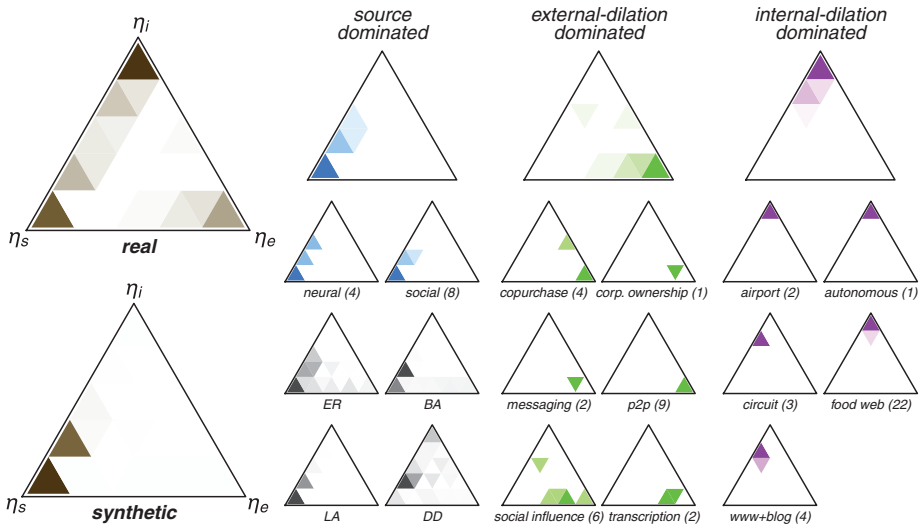


Fig. 3. Real and synthetic networks have substantially different control profiles. The coloring of breakout subfigures indicates the clustering observed in various types of real networks. Deeper shades of the heatmap indicate a greater density of networks with control profiles located in that region. Networks with $N_s = N_i = N_e = 0$ are omitted from this figure (9). The aggregated heatmaps are normalized to avoid biases induced by over- and underrepresented types of networks (see supplementary text for details). Numbers in parentheses indicate numbers of networks present in each plot.

ever, more generally, these methods lack the ability to specify a target control profile at all. Thus, in much the same way as many network generation methods have parameters to set specific degree distributions or clustering, future work on controllability in complex networks will require network models in which the control profile can be tuned to match those observed in the real world.

Real networks also have control profiles that are different from the profiles of their randomized counterparts. Figure S3 shows the impact of several randomization schemes, in particular, highlighting the loss or gain of internal dilations indicated visually by a vertical shift in the heatmap density. This suggests that many real networks have an internal dilation signature that distinguishes them from other randomized instantiations.

Although the control profiles of real networks are diverse, they also show a strong tendency to cluster around the three components of the control profile itself, suggesting that real networks broadly fall into three distinct classes: external-dilation dominated, source dominated, and internal-dilation dominated (see Fig. 3). Because these clusters do not correspond to known groupings of networks, we can test the conjecture that similar network types cluster together. These control profile clusters are statistically significant according to two separate tests for such consistency (see table S1 and fig. S2).

Although the class of external-dilation-dominated networks comprises a diverse set of systems, the control profiles of these networks consistently reflect a surplus of sinks, indicating that the systems cannot be fully controlled in a top-down manner (from sources alone). Instead, a control scheme that uses only sources will yield correlated behavior among nodes that are downstream from a common source. Such synchronized behavior may be an objective of the system. For example, in corporate-ownership, leadership, and trust hierarchies, decisions or beliefs at the sources (the owners and leaders) will drive correlated behavior, functional differentiation, and beliefs in owned organizations, followers, and advice-seekers (15, 16). Genes involved in transcriptional systems exhibit such high degrees of correlated expression that these correlations form the basis for cancer diagnosis and staging (17–19). Similarly, in copurchase networks, many products (accessories) may derive meaning, and therefore shopper relevance, from a smaller set of core products (the items that need to be accessorized). As a result, in each case above, to achieve full controllability, additional controls must be added to nonsource nodes.

Source-dominated networks, unlike the external-dilation-dominated class, have no more sinks than controls. Where an external-dilation-dominated control profile indicated a top-down organization, intended to drive correlated behavior through the system, a source-dominated control profile may suggest the opposite—a tendency toward allowing relatively uncorrelated behavior. This makes distributed processing possible, which is characteristic of the systems that fall into this class: biological neural and social networks. In both, the systems

are structured to enable independent computations and interaction at a massive scale (20, 21).

Internal-dilation-dominated networks include food networks, electrical circuits, airport interconnectivity, intranets, and the Internet's autonomous systems. These share a fundamental feature in common: They are all, more or less, closed systems. Reinforcing this idea is that many of these systems obey conservation laws, i.e., Kirchhoff's law for electrical circuits and biomass conservation within an ecosystem. Those that do not have a formal conservation law are still characterized by the circulation and recirculation of a resource (e.g., information and perspectives in the blogosphere and population movement within transportation systems) that drive the evolution of the system's state. Consistent with the notion of a closed system and conservation laws, internal-dilation-dominated networks represent systems that can be intended to function relatively independently of external stimulation.

More generally, external-dilation- and internal-dilation-dominated networks both require many controls to be attached to nonsource nodes in order to provide full controllability. When sources correspond to inputs to the system, we might interpret dilations to be the signature of a network that is not meant to be fully controlled, in that it is not designed to visit every possible system state (e.g., the conservation laws in internal-dilation-dominated networks impose constraints such that only a portion of the full state space can be explored). Indeed, in the case of transcriptional networks, electronic systems, and the Internet, certain states are pathological to or are simply unexplored by a system under natural conditions (22). A tantalizing direction for future work is to consider the extent to which internal dilations themselves reveal characteristics of forbidden regions of state space.

Nevertheless, the extent to which the control properties of networks are determined by source nodes can give insight into whether it is actually practical to control the entire system. Sources in a network can correspond to elements that lie on the boundary of the system—variables to which we have direct access. For example, in the case of biochemical pathways in human cells, target receptor proteins responsible for transducing extracellular stimuli into intracellular signals act as source nodes in the cellular signaling network (23, 24). Because these nodes are readily accessible and influence the protein interactions within the cell, a large percentage of drugs used today target these receptor proteins, rather than intracellular proteins directly, to effect change within the cell (25). Thus, networks whose control profiles are dominated by sources may have a much more feasible actuation scheme.

Finally, despite surveying a wide array of complex networks, our analysis yields few networks with profiles that contain nontrivial contributions of all three components. This suggests that many complex systems may share in common a general principle that makes such control profiles unfavorable.

Understanding the causal origin of a network's minimal control points can yield both direct and comparative insights into the system and its con-

trol properties. Certainly, the scale and organic nature of connectivity present in many complex systems suggest that strategies for controlling them will be nontrivial; thus, such fundamental insights may be crucial to such endeavors.

Here, we have identified the major structures that induce the need for controls in a complex network in order to guarantee controllability. This led to the discovery that for many networks, we can approximate the number of controls simply by the zero-degree points of the degree distribution. Subsequently, we defined the control profile, the decomposition of a complex network into the structural elements that induce the need for specific controls—sources, external dilations, and internal dilations. We find that control profiles highlight a profound and systemic difference between the control structures of random network models and real networks. Furthermore, the control profiles of real networks fall into three clusters that correspond to different aspects of high-level structure and function. Thus, the control profile is a network statistic that may prove useful in better understanding and approaching the control of complex networks.

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Supplementary Materials

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 Materials and Methods

Figs. S1 to S4
 Tables S1 and S2
 References (26–70)

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Fossilized Nuclei and Chromosomes Reveal 180 Million Years of Genomic Stasis in Royal Ferns

Benjamin Bomfleur,^{1*} Stephen McLoughlin,^{1*} Vivi Vajda²

Rapidly permineralized fossils can provide exceptional insights into the evolution of life over geological time. Here, we present an exquisitely preserved, calcified stem of a royal fern (*Osmundaceae*) from Early Jurassic lahar deposits of Sweden in which authigenic mineral precipitation from hydrothermal brines occurred so rapidly that it preserved cytoplasm, cytosol granules, nuclei, and even chromosomes in various stages of cell division. Morphometric parameters of interphase nuclei match those of extant *Osmundaceae*, indicating that the genome size of these reputed “living fossils” has remained unchanged over at least 180 million years—a paramount example of evolutionary stasis.

Royal ferns (*Osmundaceae*) are a basal group of leptosporangiate ferns that have undergone little morphological and anatomical change since Mesozoic times (1–6). Well-preserved fossil plants from 220-million-year-old rocks already exhibit the distinctive architecture of the extant interrupted fern (*Osmunda claytoniana*) (2), and many permineralized os-

mundaceous rhizomes from the Mesozoic are practically indistinguishable from those of modern genera (3–5) or species (6). Furthermore, with the exception of one natural polyploid hybrid (7), all extant *Osmundaceae* have an invariant and unusually low chromosome count (7, 8), suggesting that the genome structure of these ferns may have remained unchanged over long periods

of geologic time (8). To date, evidence for evolutionary conservatism in fern genomes has been exclusively based on studies of extant plants (9, 10). Here, we present direct paleontological evidence for long-term genomic stasis in this family in the form of a calcified osmundaceous rhizome from the Lower Jurassic of Sweden with pristinely preserved cellular contents, including nuclei and chromosomes.

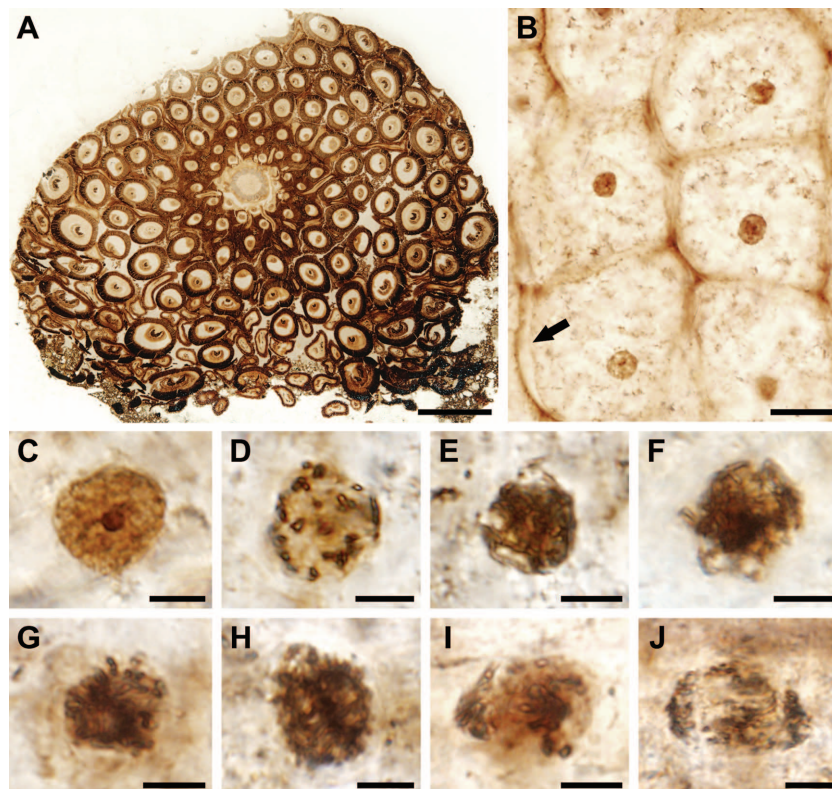
The specimen was collected from mafic volcanoclastic rocks [informally named the “Djupadal formation” (11)] at Korsaröd near Höör, Scania, Sweden [fig. S1 of (12)]. Palynological analysis indicates an Early Jurassic (Pliensbachian) age for these deposits (11) (fig. S2), which agrees with radiometric dates obtained from nearby volcanic necks (13) from which the basaltic debris originated. The fern rhizome was permineralized in vivo by calcite from hydrothermal brines (11, 14) that per-

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Fig. 1. Cytological features preserved in the apical region of the Korsaröd fern fossil. (A) transverse section through the rhizome; (B) detail of radial longitudinal section showing typical pith-parenchyma cells with preserved cell membranes (arrow), cytoplasm and cytosol particles, and interphase nuclei with prominent nucleoli; (C) interphase nucleus with nucleolus and intact nuclear membrane; (D) early prophase nucleus with condensing chromatin and disintegrating nucleolus and nuclear membrane; (E and F) late prophase cells with coiled chromosomes and with nucleolus and nuclear membrane completely disintegrated; (G and H) prometaphase cells showing chromosomes aligning at the equator of the nucleus; (I and J) possible anaphase cells showing chromosomes attenuated toward opposite poles. (A), (C to E), (G), and (I) are from NRM S069656. (B), (F), (H), and (J) are from NRM S069658. Scale bars: (A) 500 μ m; (B) 20 μ m; (C to J) 5 μ m.



colated through the coarse-grained sediments shortly after deposition (table S1). The fossil is 6 cm long and 4 cm wide and consists of a small (~7 mm diameter) central stem surrounded by a dense mantle of persistent frond bases with interspersed rootlets (Fig. 1). Its complex reticulate vascular cylinder (ectophloic dictyoxyle siphonostele), parenchymatous pith and inner cortex, and thick fibrous outer cortex are characteristic features of Osmundaceae (1, 3–5, 12) (fig. S3). Moreover, the frond bases mantling the rhizome contain a heterogeneous sclerenchyma ring that is typical of extant *Osmunda* sensu lato (1, 3, 4, 12) (fig. S4). The presence of a single root per leaf trace favors affinities with (sub)genus *Osmundastrum* (1, 3, 6, 12).

The specimen is preserved in exquisite subcellular detail (Fig. 1 and figs. S4 and S5). Parenchyma cells in the pith and cortex show preserved cell contents, including membrane-bound cytoplasm, cytosol granules, and possible amyloplasts (Fig. 1 and fig. S5). Most cells contain interphase nuclei with conspicuous nucleoli (Fig. 1, figs. S4 and S5, and movies S1 and S2). Transverse and longitudinal sections through the apical part of the stem also reveal sporadic dividing parenchyma cells, mainly in the pith periphery (Fig. 1). These are typically preserved in prophase or telophase stages, in which the nucleolus and nuclear envelope are more or less unresolved and the chromatin occurs in the form of diffuse, granular material or as distinct chromatid strands. A few

cells contain chromosomes that are aligned at the equator of the nucleus, indicative of early metaphase, and two cells were found to contain chromosomes that appear to be attenuated toward opposite poles, representing possible anaphase stages. Some tissue portions in the stem cortex and the outer leaf bases show signs of necrosis and programmed cell death (fig. S6).

Such fine subcellular detail has rarely been documented in fossils (15–17) because the chances for fossilization of delicate organelles are small (16) and their features are commonly ambiguous (17). The consistent distribution and architecture of the cellular contents in the Korsaröd fern fossil resolved via light microscopy (Fig. 1 and fig. S4), scanning electron microscopy (fig. S5), and synchrotron radiation x-ray tomographic microscopy (SRXTM) (fig. S5 and movies S1 and S2) provide unequivocal evidence for three-dimensionally preserved organelles.

Positive scaling relationships rooted in DNA content can be used to extrapolate relative genome sizes and ploidy levels of plants (18–21). We measured minimum and maximum diameters, perimeters, and maximum cross-sectional areas of interphase nuclei in pith and cortical parenchyma cells of the fossil and of its extant relative *Osmundastrum cinnamomeum*. The measurements match very closely (Fig. 2), with mean nuclear perimeters of 32.2 versus 32.6 μm and mean areas of 82.2 versus 84.9 μm^2 in the fossil

and in extant *Osmundastrum*, respectively. The equivalent nuclear sizes demonstrate that the Korsaröd fern fossil and extant Osmundaceae likely share the same chromosome count and DNA content, and thus suggest that neither ploidyization events nor notable amounts of gene loss have occurred in the genome of the royal ferns since the Early Jurassic ~180 million years ago [(8), see also discussion in (9, 10)]. These results, in concert with morphological and anatomical evidence (1–6), indicate that the Osmundaceae represents a notable example of evolutionary stasis among plants.

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S6

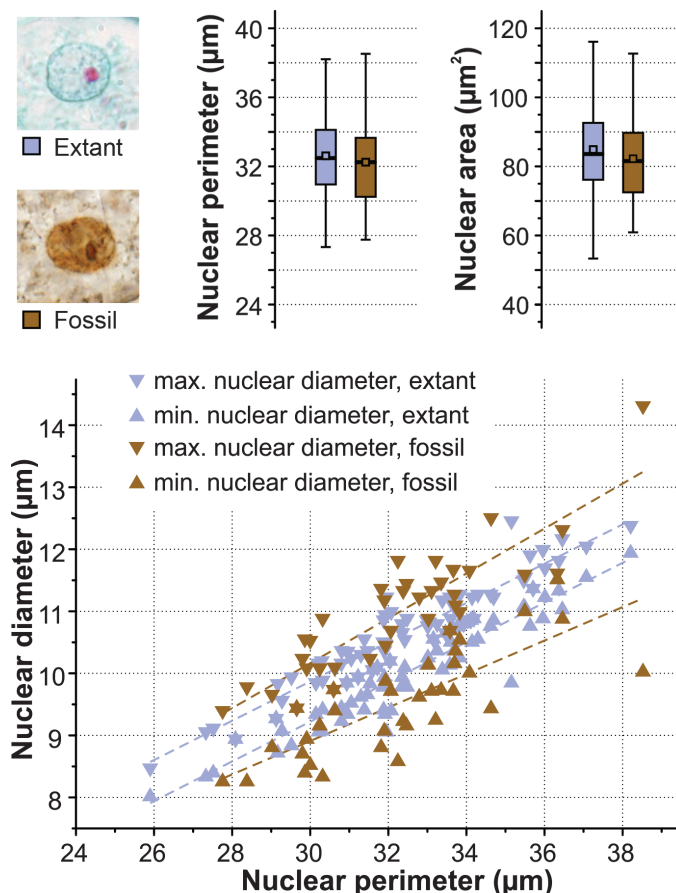
Table S1

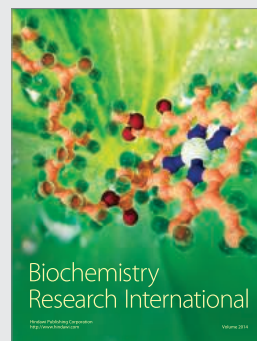
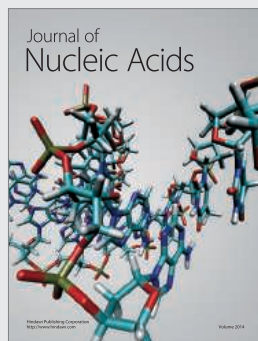
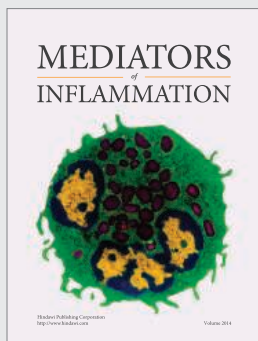
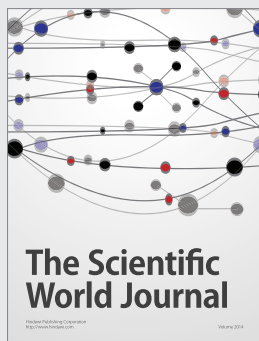
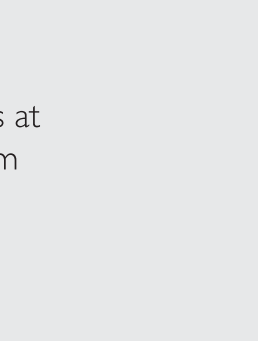
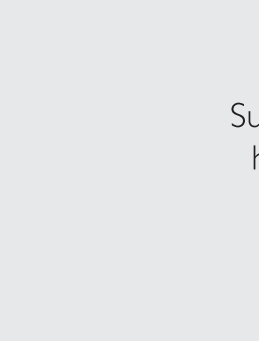
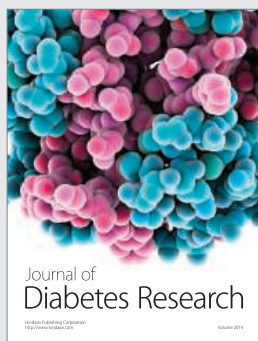
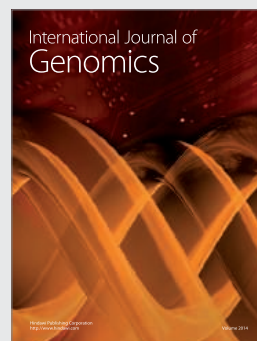
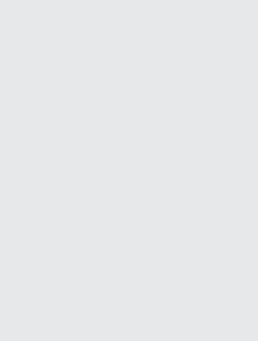
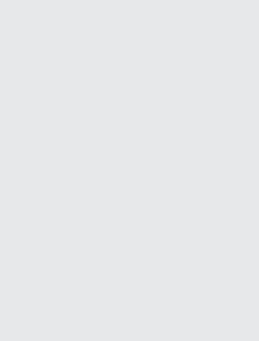
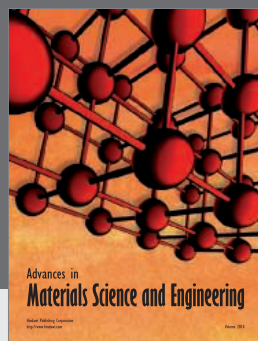
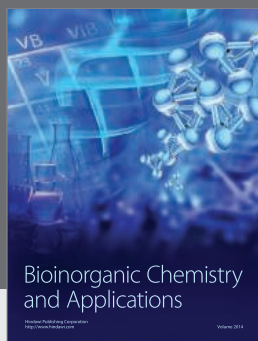
References (22–36)

Movies S1 and S2

17 December 2013; accepted 21 February 2014
10.1126/science.1249884

Fig. 2. Morphometric parameters of interphase nuclei of extant *O. cinnamomeum* compared to those of the Korsaröd fern fossil. Colored box-and-whiskers plots in upper graph indicate interquartile ranges (box) with mean (square), median (solid transverse bar), and extrema (whiskers); dashed colored lines in lower graph indicate linear fits ($n = 76$ versus $n = 37$ measured nuclei for extant *O. cinnamomeum* versus the fossil).





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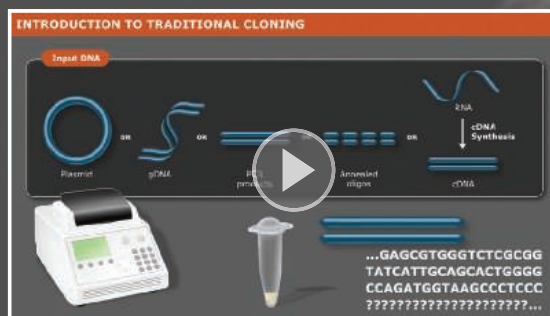
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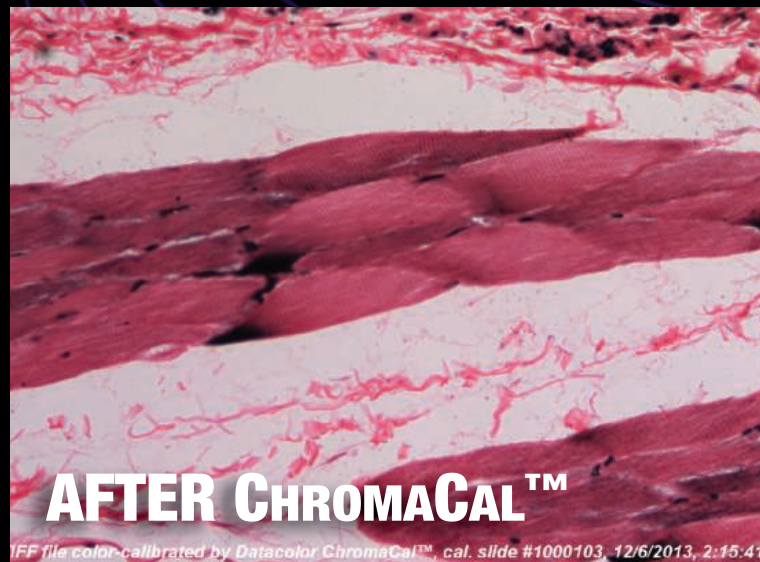
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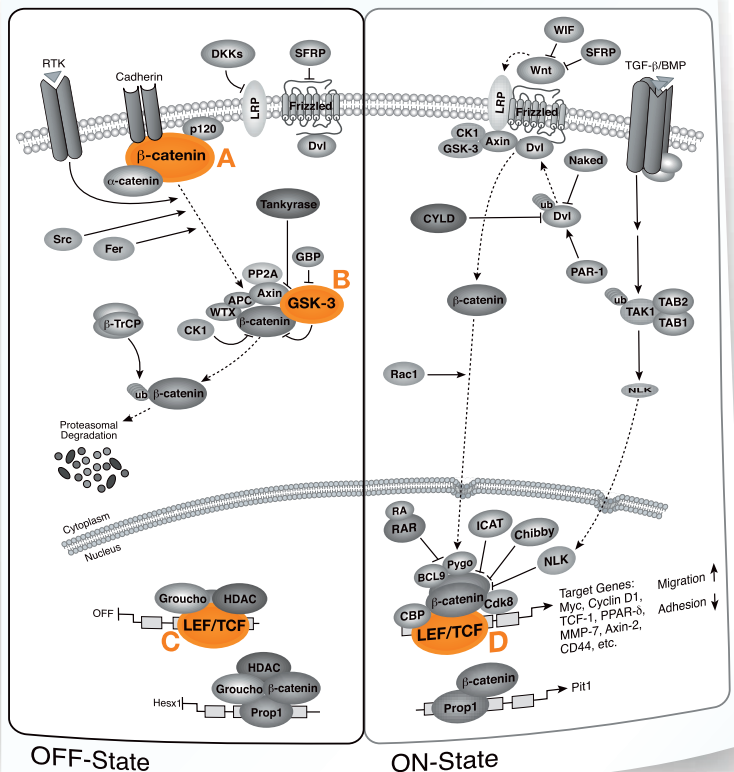
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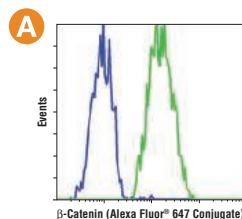
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Wnt/ β -Catenin Signaling

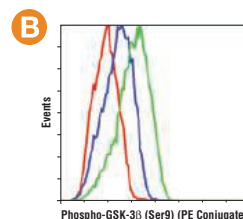


#4627 detects β -Catenin in HeLa (positive), but not NCI-H28 (negative) cells.

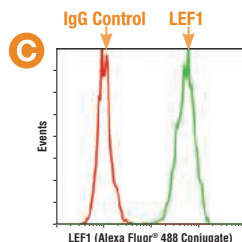
#8466 detects Ser9 phosphorylated GSK-3 β in stimulated cells.



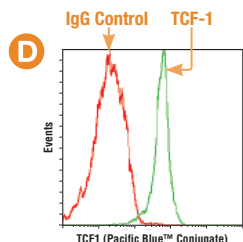
β -Catenin (L54E2) Mouse mAb (Alexa Fluor[®] 647 Conjugate) #4627: Analysis of NCI-H28 cells (blue) and HeLa cells (green).



Phospho-GSK-3 β (Ser9) (D85E12) XP[®] Rabbit mAb (PE Conjugate) #8466: Analysis of NIH/3T3 cells treated with hPDGF-BB #8912 and λ phosphatase (red), untreated (blue), or treated with hPDGF-BB #8912 only (green).



LEF1 (C12A5) Rabbit mAb (Alexa Fluor[®] 488 Conjugate) #8490: Analysis of Jurkat cells using LEF1 (C12A5) Rabbit mAb (Alexa Fluor[®] 488 Conjugate) (green) compared to Rabbit (DA1E) mAb IgG XP[®] Isotype Control (Alexa Fluor[®] 488 Conjugate) #2975 (red).

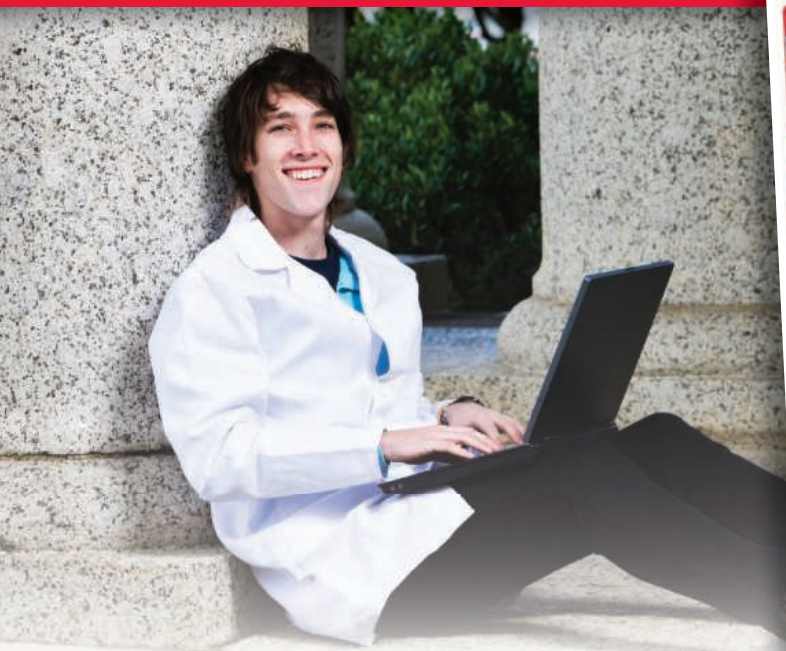


TCF1 (C63D9) Rabbit mAb (Pacific Blue[™] Conjugate) #9066: Analysis of Jurkat cells using TCF1 (C63D9) Rabbit mAb (Pacific Blue[™] Conjugate) (green) compared to Rabbit (DA1E) mAb IgG XP[®] Isotype Control (Pacific Blue[™] Conjugate) #9078 (red).



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THE CHINESE UNIVERSITY OF HONG KONG

Applications are invited for:-

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Associate Professor(s) / Assistant Professor(s) (Non-clinical)

(Ref. 1314/129(665)/2) (Closing date: April 17, 2014)

The School, established in the Faculty of Medicine in June 2009, has the vision of becoming a world-recognized leader in selected fields of biomedical sciences while maintaining excellence in postgraduate and undergraduate education, with research programmes currently focusing on neuro-degeneration, cancer biology and inflammation, developmental biology, reproduction and endocrinology, vascular and metabolic biology and stem cell and regeneration.

Individuals who are strongly motivated by the biological importance of their research and who value the opportunity to work in close collaboration with basic and clinical scientists are welcome. Applicants should have (i) a PhD, MD or DVM degree or equivalent in biological and biomedical sciences or related disciplines; (ii) experience in using cutting-edge approaches such as bioinformatics, genomics, molecular genetics, stem cells, transgenic animal and imaging technologies; and (iii) a good track record of research and publications in international peer-reviewed scientific journals in one or more related areas. Those with expertise in human genetics, neurodegenerative diseases, stem cells biology, mammalian development and regeneration biology are particularly welcome.

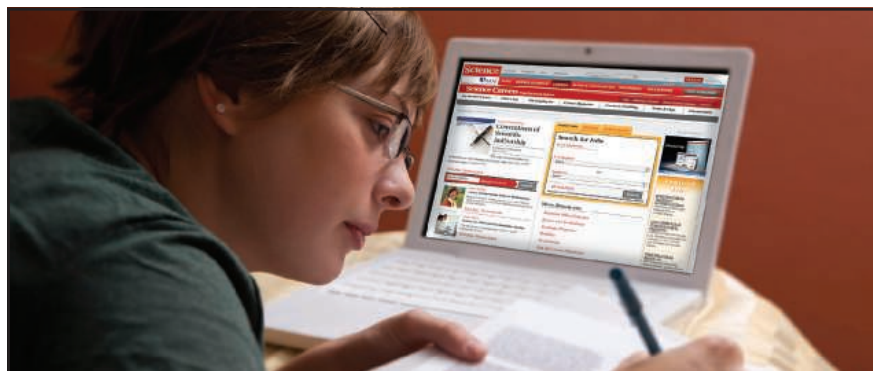
Duties include (a) teaching undergraduate, postgraduate and general education courses; and (b) leading a vigorous independent research programme. Appointee(s) will be provided with appropriate laboratory and office space and a start-up package commensurate with qualifications and experience. Appointment(s) will normally be made on contract basis for up to three years initially commencing in the third quarter of 2014 or thereafter, which, subject to mutual agreement, may lead to longer-term appointment or substantiation later.

Salary and Fringe Benefits

Salary will be highly competitive, commensurate with qualifications and experience. The University offers a comprehensive fringe benefit package, including medical care, a contract-end gratuity for appointments of two years or longer, and housing benefits for eligible appointees. Further information about the University and the general terms of service for appointments is available at <http://www.per.cuhk.edu.hk>. The terms mentioned herein are for reference only and are subject to revision by the University.

Application Procedure

Please send full resume, together with copies of academic credentials, a publication list and/or abstracts of selected published papers, and names, addresses and fax numbers/e-mail addresses of three referees to whom the applicants' consent has been given for their providing references (unless otherwise specified), to the Personnel Office, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong [fax: (852) 3943 1462] by the closing date. The Personal Information Collection Statement will be provided upon request. Please quote the reference number and mark 'Application – Confidential' on cover.



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A New Approach to Engineering

The Institute for Molecular Engineering is a unique interdisciplinary program launched in 2011 by the University of Chicago, in partnership with Argonne National Laboratory. It was created with the aim of translating molecular-level science into technological solutions and engineering systems with potential societal impact in energy, health care, environmental sustainability, information technology, and more.

This year, the institute is adding four prominent senior faculty members: Andrew Cleland from the University of California, Santa Barbara; Giulia Galli from the University of California, Davis; and Melody Swartz and Jeffrey Hubbell from the École Polytechnique Fédérale de Lausanne. At the University of Chicago, these professors will lead programs in the areas of cancer research, bioengineering for immunotherapy, novel materials for water resource management, quantum computing, and quantum materials—and develop new approaches to engineering research and education.

The Institute for Molecular Engineering continues to invite applications for faculty positions at all ranks. Interested applicants must apply online at academiccareers.uchicago.edu, posting number 01983.

Learn more at ime.uchicago.edu.



ANDREW CLELAND

Professor in Molecular Engineering

Associate director of the California Nanosystems Institute at the University of California, Santa Barbara, Cleland is at the forefront of applying quantum mechanics, having led the team that created the first quantum machine.



GIULIA GALLI

Liew Family Professor in Molecular Engineering

Former chair of the Extreme Physics and Chemistry of Carbon Directorate of the Deep Carbon Observatory, Galli is a pioneer in utilizing theoretical and computational methods to predict the properties of complex materials and identify new materials with targeted properties.



JEFFREY HUBBELL

Professor in Molecular Engineering

Merck-Serono Chair in Drug Delivery at the École Polytechnique Fédérale de Lausanne, Hubbell is an entrepreneurial materials scientist, developing biomaterials with great promise in the fields of regenerative medicine and treatment delivery.



MELODY SWARTZ

Professor in Molecular Engineering

Director of the Institute of Bioengineering at the École Polytechnique Fédérale de Lausanne, Swartz is redefining how other scientists regard the lymphatic system and its relationship to the cell transport system with the goal of improving cancer immunotherapy.

CONAGEN

Conagen-Aiitech is a growing R&D company located in Boston, Massachusetts. Established in 2009, we focus on using synthetic biology and metabolic engineering techniques to produce high-value ingredients for food, flavor, fragrance, and pharmaceutical industries. We are currently seeking a number of senior scientists and lab technicians to participate in several on-going projects:

- 1) Five **senior fermentation scientists** including a **Chief Fermentation Engineer**. They will join a team of engineers and scientists to perform new factory design and operation, fermentation process scale up and optimization, or establishment of fermentation processes for the production of a variety of biochemicals.
- 2) Two senior computational biologists. **One** will focus on exploring existing and newly generated databases for metabolic pathway discovery, and bioinformatics-based enzyme function prediction. **The other** will focus on design, development, and data integration of a laboratory automation system anchored by an automatic liquid handling system.
- 3) **One senior protein scientist** to focus on protein structure analysis and modeling for the purpose of protein engineering. The candidate shall have experience altering enzyme activities through protein manipulations.
- 4) **One senior biochemist** to focus on enzyme discovery and functional analysis. We are interested in candidates with experience in plant/fungal/bacterial cell wall biochemistry or secondary metabolisms.
- 5) Four **laboratory technicians** to support the above mentioned projects.

If you are interested, please visit the career pages at <http://www.conagen-inc.com> for more information on application requirements and procedures.



Technical University of Denmark



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DTU is a technical university providing internationally leading research, education, innovation and public service. Our staff of 5.000 advance science and technology to create innovative solutions that meet the demands of society; and our 10.000 students are educated to address the technological challenges of the future. DTU is an independent academic university collaborating globally with business, industry, government, and public agencies.

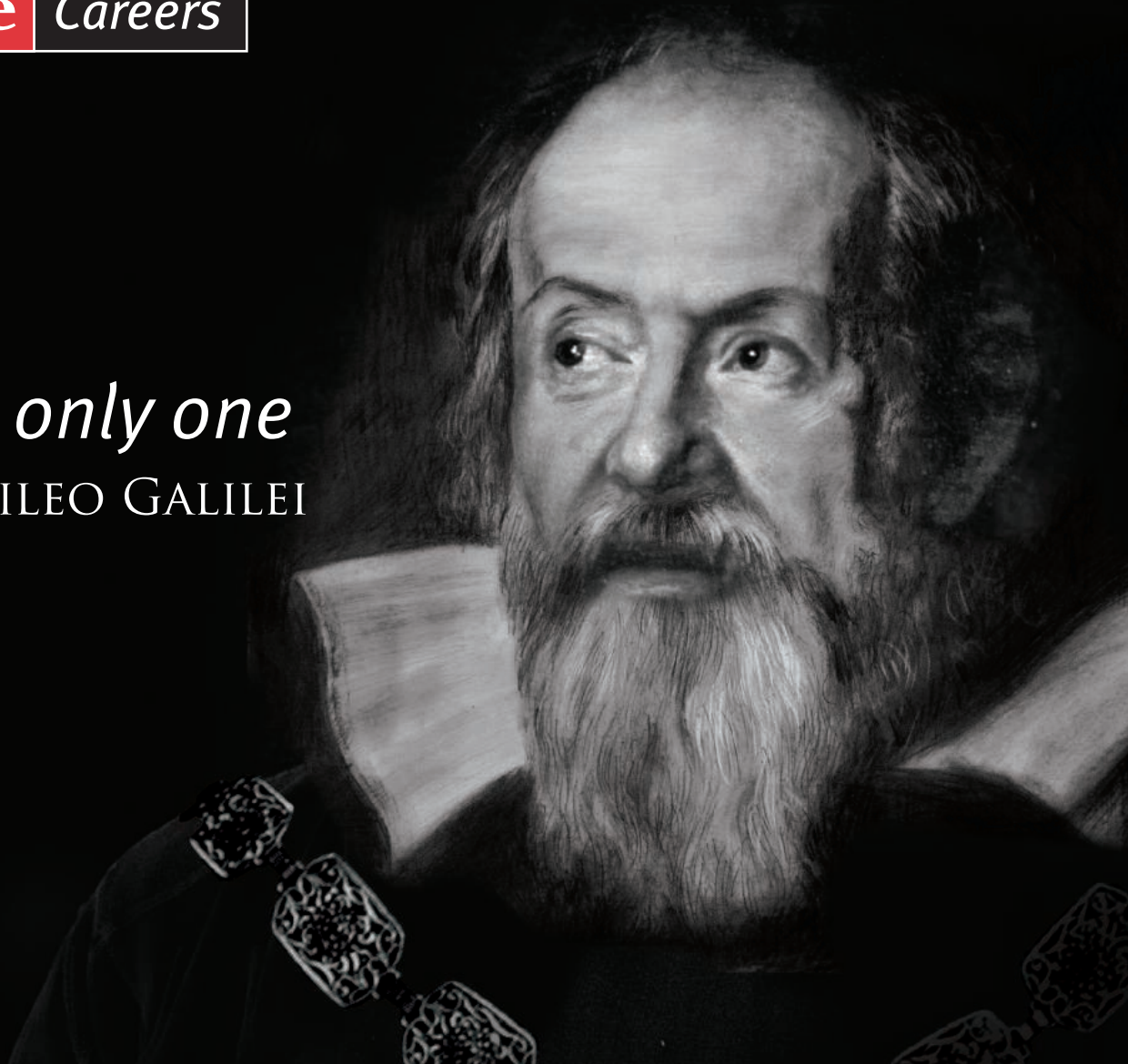
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Born in 1564, Galileo Galilei once contemplated a career in the priesthood. It's perhaps fortunate for science that upon the urging of his father, he instead decided to enroll at the University of Pisa. His career in science began with medicine and from there he subsequently went on to become a philosopher, physicist, mathematician, and astronomer, for which he is perhaps best known. His astronomical observations and subsequent improvements to telescopes built his reputation as a leading scientist of his time, but also led him to probe subject matter counter to prevailing dogma. His expressed views on the Earth's movement around the sun caused him to be declared suspect of heresy, which for some time led to a ban on the reprinting of his works.

Galileo's career changed science for all of us and he was without doubt a leading light in the scientific revolution, which is perhaps why Albert Einstein called him the father of modern science.

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南开大学
Nankai University

"National Thousand (Young) Talents Program", "Chang Jiang Scholars" and other faculty positions available in Nankai University

Nankai University locates in Tianjin, the third largest city in China and 30 min away from Beijing by a high-speed train. Nankai University is one of the key national universities directly under the jurisdiction of the Ministry of Education of China, and also a "211 Project" and "985 Project" university in China. Nankai University is the center for both education and academic research, and has materialized a series excellent achievements, with the quality and the quantity of the research papers, projects, funds, and prizes among the forefront of the national universities of China. Premier Enlai Zhou, the world-wide known mathematician Shiing-shen Chern, physicist Ta-you Wu and playwright Yu Cao are alumni of Nankai University. Nankai University is recruiting for outstanding investigators to occupy the following honorable positions.

1. Professors and Associate Professors of "The National Thousand (Young) Talents Program", "The State (Young) Special Support Plan", and other high-level talent programs: In addition to the requirements defined by the program, such as "The National Thousand Talent Program" (<http://www.1000plan.org/>), the applicant with good health conditions should be a well-established and highly innovative scientist with strong academic records and leadership. The applicant for a young program should be able to demonstrate the potency to be an outstanding scientist in the future with the support from Nankai University.

2. Permanent and visiting professors of "Chang Jiang Scholars Program": In addition to the requirements defined by the program (please see: <http://www.changjiang.edu.cn/>), the successful applicant with good health conditions should be an internationally known investigator with well-known achievements in the field, a strong leadership in guiding a first-class research team and a high capability in management and organization.

3. Nankai University Hundred Young Academic Leaders Program

This program targets the excellent young scholars who are less than 40 years old in humanity, social sciences and natural sciences fields, breaking the title barrier in application. The applicants can apply for this program before or after officially working in Nankai University and the selected scholars will be provided all-round support, such as performance-based pay of the highest level of professors, research fund, experiment and working conditions.

4. Other positions (Professor/Associate Professor/Lecturer/Postdoctoral Researchers/Nankai Lecture Professor)

Please visit <http://www.nankai.edu.cn/s/12/t/27/47/27/info18215.htm> for more information.

Salary, start-up package and benefits: The recruited faculty at different academic levels will be supported with competitive salary, the start-up package (competitive start-up funds, newly renovated office/lab and experienced assistants), housing allowance, medical insurance and other possible benefits. All of the above offers are negotiable.

Contact us: Applicants should send curriculum vitae in both English and Chinese, the first page of 5 publications, statement of research interests/plans and at least three references to: Ms. Yang Liu and/or Mr. Yuechao Wang, Office of Human Resources, Nankai University, 94 Weijin Road, Tianjin, China, 300071; Tel(Fax): 0086-22-2350-8595; Personnel Office website: <http://rsc.nankai.edu.cn>; Email: nkuniversity@nankai.edu.cn.

"Hundred Young Academic Leaders Program" is now open for application, and please visit <http://employment.nankai.edu.cn/WebHR> for more information. You will be contacted once we receive and finish the evaluation of your application for the position.

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Xuzhou Medical College Recruitment Plan for Jiangsu specially-appointed professors in 2014

Xuzhou Medical College, situated in the city of Xuzhou, was founded in 1958. It is now the center of medical education, medical service and medical research in Northern Jiangsu and in the whole Huaihai economic zone.

I. Disciplines for Application: Biology, Basic Medicine, Pharmacy, Clinical Medicine, Public Health and Preventive Medicine, Stomatology.

II. Requirements for overseas applicants: (1) All applicants should have earned a doctoral degree and ideally they should be no more than 45 years old (born after January 1st, 1969). (2) All applicants should have a minimum of two consecutive years of experience in undertaking research overseas after obtaining their doctoral degrees. More specifically, they must have been conferred with at least the title of Assistant Professor, or a position of similar status, in a recognized and accredited non-Chinese university or research institution.

III. Duration of Employment and Benefits: The Jiangsu Provincial Government will provide 1,000,000.00-2,000,000.00 RMB as research funding for Jiangsu specially-appointed professors. Xuzhou Medical College will provide research funding which will be no lower than the Provincial standard. For the 3-year employment period, the Jiangsu Provincial Government will provide a bonus of 120,000.00 RMB for each professor every year. Xuzhou Medical College will also give competitive offers.

IV. More information and to apply visit website:

<http://rsc.xzmc.edu.cn/>

V. Contact:

Address: No. 209 Tongshan Rd. Xuzhou, Jiangsu province, China (postal code: 221004)

Department: Personnel Department, Xuzhou Medical College

Contacts: Du Gang, Li Yuandong

Tel: 0516-83262040, 83262042

Email: szb@xzmc.edu.cn

Website: www.xzmc.edu.cn

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Neuroscience Gene Vector and Virus Core Director

Stanford University is seeking an outstanding and entrepreneurial molecular biologist/virologist to lead and manage the Neuroscience Gene Vector and Virus Core (<http://med.stanford.edu/gvvc>). This core assists in the design of viral vectors and generation of viral stocks for Stanford researchers. The successful candidate will have Ph.D. and/or M.D., and several years of productive postdoctoral research involving the generation and use of viruses (e.g., adeno associated viruses (AAV), lentiviruses, and retroviruses). Experience and knowledge of AAV biology is strongly desired. The independent director supervises a team of molecular biologists/virologists, is responsible for the administration and operation of the core, and works with School of Medicine Staff to set service rates and develop the core business strategy. Salary will be commensurate with experience.

Please submit a CV, statement of research experience, and have 3 letters of recommendation sent by April 15, 2014 to:

Kristy Verhines, ksn@stanford.edu

Stanford University is committed to increasing representation of women and members of minority groups on its staff and particularly encourages applications from such candidates.

THE UNIVERSITY OF NIGERIA NSUKKA

INTERNATIONAL CENTRE FOR BIOTECHNOLOGY

A Centre under the auspices of UNESCO

Executive Director of the International Centre of Biotechnology at the University of Nigeria Nsukka (ICB – UNN) a UNESCO Category II Centre

The newly founded International Centre for Biotechnology under the auspices of UNESCO, located in the university town of Nsukka, in South-Eastern Nigeria, is seeking a highly motivated Director to drive its launch and subsequent growth.

The ideal candidate for the post should have an excellent track record of research and publication in a relevant field in the area of Biotechnology and at least five years' experience either as a senior researcher or Director of an equivalent institute. In addition, the candidate should possess proven managerial and interpersonal skills, which will be deployed in overseeing the overall research activities of the Institute, fostering productive partnerships with collaborators and stakeholders and the attraction of resources through research grants and other fund-raising activities, which may be needed for the sustenance and growth of the Institute.

As the first Director of the Institute, you will play a vital role in driving the strategic vision of the Institute and will have a responsibility for ensuring that the Institute becomes internationally recognized as one of the premier research centres in Africa. You will be responsible for ensuring that the Institute achieves international recognition as a regional centre for innovative research in the mandate areas of Food Security, Bio-resource Conservation and Tropical Disease.

Candidates must hold a PhD or an equivalent title in a relevant field of study, such as Molecular Biology, Pharmacology, Biochemistry, Microbiology and Medicine, and should be actively involved in research.

Candidates will be offered a competitive salary and conditions of service, and will receive a relocation allowance.

Additional information on this post can be found at: <http://www.unesco.org/new/ICB-UNN>

Applicants should send a cover letter, CV with full list of publications, a concise description of concept of the development and management of ICB-UNN and a statement of research interest to: l.hoareau@unesco.org. Shortlisted candidates will be invited for a presentation at ICB-UNN during a special meeting of the International Advisory Board in the spring of 2014.

For more information, please contact Prof. Bartholomew N. Okolo, Chair of the ICB-UNN International Advisory Board (bato_okolo@yahoo.com), Prof. Jerry Ugwuanyi, Project Coordinator, ICB-UNN (phone: +234-8033066518; Email: jerry.ugwuanyi@unn.edu.ng) or Professor Maciej J. Nalecz, Director of the International Basic Sciences Programme, UNESCO (m.nalecz@unesco.org).



www.jobs.cam.ac.uk

Lucasian Professorship of Mathematics

Department of Applied Mathematics and Theoretical Physics

The Board of Electors to the Lucasian Professorship of Mathematics invite applications for this Professorship to take up appointment on 1 September 2014, or as soon as possible thereafter, from persons whose work falls in the general fields of applied mathematics and theoretical physics.

Candidates will have an outstanding international reputation in their field of research and will be expected to provide strong academic leadership in research, teaching and other activities of the Department. They will hold a PhD or equivalent postgraduate qualification.

Standard professorial duties include teaching and research, examining, supervision of graduate students and administration. The Professor will be based in Cambridge. A competitive salary will be offered.

Further information is available at: www.admin.cam.ac.uk/offices/academic/secretary/professorships/ or contact the Academic Secretary, University Offices, The Old Schools, Cambridge CB2 1TT, (email: ibise@admin.cam.ac.uk).

Applications, consisting of a letter of application, a statement of current and future research plans, a curriculum vitae and a publications list, along with details of three referees should be made online no later than Tuesday 15 April 2014.

Women are currently under-represented in Professorial posts in the University; therefore we particularly welcome applications from women for this post.

Informal enquiries may be made to Professor Peter Haynes, Head of the Department of Applied Mathematics and Theoretical Physics, telephone +44 (0)1223 337862 or email phh1@cam.ac.uk

Please quote reference LE02923 on your application and in any correspondence about this vacancy.

Closing date: 15 April 2014.

The University values diversity and is committed to equality of opportunity.

The University has a responsibility to ensure that all employees are eligible to live and work in the UK.



BIOCHEMISTRY FACULTY POSITION

The Department of Molecular and Cellular Biochemistry at the University of Kentucky invites applications for a tenure track faculty position at the ASSISTANT, ASSOCIATE or FULL professor level. We are particularly interested in candidates whose area of research complements existing departmental strengths including, but not limited to metabolomics, chemical biology, and the molecular basis of cardiovascular disease, neurodegenerative disorders, diabetes, and cancer. The successful candidate must possess a Ph.D., M.D. or equivalent degree. Senior applicants must have an active, internationally recognized, extramurally funded research program. For junior applicants, possession of extramural funding is strongly preferred. Translational programs that encompass aspects of both basic and clinical research are of special interest.

The successful candidate will benefit from a stimulating and collaborative environment within the department, a strong graduate program, and state-of-the-art facilities in a new 185,000 ft² research building. Competitive start-up funds and appropriate space will be offered.

Evaluation of applicants will begin **April 1, 2014**, and continue until the position is filled. Interested applicants should visit <http://medicine.mc.uky.edu/biochemreference> to access the on-line faculty application. Required application materials include a curriculum vitae, a description of your current and future research program, and the names of three references. Inquiries may also be directed to **Dr. Rebecca Dutch**, Chair, Faculty Search Committee at biochem@uky.edu.

For further information about the Department, visit: <http://biochemist.med.uky.edu/>

The University of Kentucky is an Equal Opportunity Employer and encourages applications from minorities and women.

**EXECUTIVE DIRECTOR****Division of Earth and Ecosystem Sciences**

The Desert Research Institute (DRI) has an exciting opportunity for an Executive Director for its Division of Earth and Ecosystem Sciences who has a highly diversified research portfolio that includes biological, geological, and archaeological sciences. The Executive Director's primary function is to manage, facilitate, and grow divisional research activities, faculty, and staff.

Applicants must have a Ph.D. or equivalent graduate degree in archaeological, biological, Earth sciences, or a related field, or professional experience commensurate with an advanced degree. For complete details, visit **website: <http://jobs.dri.edu>**. DRI is an Affirmative Action/Equal Employment Opportunity/Disability/Veteran Employer.

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Colorado State University's (CSU) School of Global Environmental Sustainability (SoGES) seeks an Associate Director. The successful candidate will help the Director assure the School functions effectively, and takes the lead as a key player and partner in university-wide, regional, and international sustainability research and education initiatives. Minimum requirements include an Associate to Full Professor level OR advanced degree(s) plus six years relevant experience, related to sustainability sciences; evidence of significant contribution to successful, large scientific initiatives; successful fundraising or grantsmanship; scholarly accomplishments; strategic planning and long-term project coordination experience; and excellent communication skills and ability to work with diverse groups. Read full job description and apply at **website: <http://sustainability.colostate.edu/jobs>**. For full consideration, apply by March 31, 2014. CSU is an Equal Opportunity/Equal Access/Affirmative Action Employer and conducts background checks on all final candidates.

FOREST GENETICS RESEARCH LEADER

The Washington State Department of Natural Resources is seeking candidates in the fields of molecular genetics, microbiology, mycology, dendrology, or related field to fill a new research position. This position will serve as an expert in the field of forest genetics, independently performing original scientific research to improve the health and productivity of native conifer trees. The position resides in Tumwater, Washington, and includes a monthly salary of up to \$7,333 (depending on qualifications), benefits package, and potential moving expense allowance. For information contact us by **telephone: 360-902-1228** or go to our jobsite at **website: <http://www.dnr.wa.gov/AboutDNR/Employment/Pages/Home.aspx>** scroll down to Forest Genetics Research Leader announcement.

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